The local and systemic angiogenic and immunological responses to surgery

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Francis P.K. Wu

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THE LOCAL AND SYSTEMIC ANGIOGENIC AND IMMUNOLOGICAL RESPONSES TO SURGERY

ACADEMISCH PROEFSCHRIFT

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door

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geboren te Hong Kong

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copromotor: dr. K. Hoekman

Aan mijn ouders

Contents

Chapter 1

General Introduction

Angiogenesis is the process in which new blood vessels are formed from pre-existing vessels. The purpose of angiogenesis is to provide oxygen and nutrient and to remove waste products. This process is essential in embryogenesis, and in healthy adults during the menstrual cycle and healing wounds. Tumor growth beyond 2-3 mm and the development of mestastases are also angiogenesis dependent.[1-3] Angiogenesis is a complex and highly co-ordinated process and thought to be regulated by an angiogenic balance, which essentially favors pro-angiogenic growth factors (stimulators) over anti-angiogenic growth factors (inhibitors). Locally activated stromal cells produce these angiogenic factors and recruited immune cells in wound healing. During tumor growth, tumor cells are an additional and a continuous source of angiogenic factor production. These angiogenic factors are also responsible for the recruitment of circulating endothelial progenitors (CEPs) from the bone marrow, which contribute to local angiogenesis in different amounts, depending on the demand and local situation. An impressive number of pro- and anti-angiogenic factors have been identified so far, which confirms the complexity of this process (Table 1). Besides angiogenic factors, a variety of proteases are also involved in angiogenesis. Among them, the family of metalloproteases (MMPs) and plasminogen activators (PAs) and their inhibitors are found to be important players in angiogenesis. Proteases facilitate the invasion and migration of endothelial cells (ECs), but by the very process of degradation of matrix proteins they may also generate protein fragments which have anti-angiogenic potential (e.g. endostatin). Finally, activated endothelial cells express a variety of adhesion proteins (integrins, cadherins), that are essential for the communication of endothelial cells with extracellular matrix proteins (ECMs). Together, this results in proliferation, migration and tube formation of endothelial cells. Maturation of these newly formed vessels is achieved by recruitment of smooth muscle cells (pericytes), which cover these vessels. In wound healing the acute generation of cell damage and local hypoxia and acidity initiates the process of wound

healing. Although the understanding of this complex process is growing, the fundamental aspects of wound healing have not been completely been characterized as yet.

Table 1

Pro-angiogenic factors	Anti-angiogenic factors
Vascular endothelial growth factor (VEGF)	Endostatin
Basic fibroblast growth factor (bFGF)	Collagen IV fragments
Platelet-derived endothelial cell growth factor (PD-ECGF)	Angiostatin
Transforming growth factor (TGF-α & β)	Thrombospondins (TSP-1 & 2)
Epidermal growth factor (EGF)	Tumor Necrosis Factor-α (TNF-α)
Insulin-like growth factor (IGF)	Interleukins (IL-10, 12)
Hepatocyte growth factor/ Scatter factor (HGF/SF)	Interferon (IFN-α, β and γ)
Granulocyte-macrophage colony growth factor (GM-CSF)	Plasminogen activator inhibitors (PAIs)
Interleukins (IL-1, 4, 6, 8 & 15)	Tissue inhibitors of MMP (TIMP-1/-2)
Tumor Necrosis Factor-α (TNF-α)	Transforming growth factor (TGF-β)
Angiogenin	Platelet Factor-4
Angiotensin	Thrombin Antithrombin Complex
Angiotropin	Soluble VEGF-receptor-1
Fibrin	
Fibronectin	
Matrix metalloproteinases	

THE THREE PHASES OF WOUND HEALING

The wound healing process proceeds in three phases that overlap in time.

These are: 1. Hemostasis and inflammation

2. Proliferation and granulation

3. Scar tissue remodeling.[3]

Angiogenesis is an essential component in phase 1 and 2.

Wound healing requires a complex control of biological events involving immunological and cellular reaction cascades, angiogenesis, production of extracellular matrix (ECM) proteins and cytokines.

Cytokines are a large group of non-enzymatic proteins that sophisticatedly co-ordinate communications between target cells. Nearly all nucleated cells are capable of producing these proteins in response to intrinsic (autoimmunity) or extrinsic (infection) responses or wound healing. A common pathway in immune responses is the initial and immediate production of interleukin-1 (IL-1) and tumor necrosis factor-α (TNF-α), followed after 6-12 hours by interleukin-6 (IL-6) and interleukin-8 (IL-8). The local release of these cytokines will either directly or indirectly control the wound healing phases. It is the balance of various cytokines that play a pivotal role in regulating the initiation, progression and completion of wound healing.

ANGIOGENIC CYTOKINES

Cytokines that are associated with angiogenesis during wound healing are discussed here.

IL-1 and TNF-α

Both IL-1 and TNF-α level have been characterized by their quick systemic appearance and disappearance after surgery, due to their half-life in the circulation, which is respectively 6 and 20 minutes.[4,5] The primary sources of IL-1 after wounding are keratinocytes,

macrophages and endothelial cells, and TNF-α is also released by keratinocytes, neutrophils, monocytes/macrophages and T-cells.[6]

Both IL-1 and TNF-α are locally and systemically active. Abundant production of pro-inflammatory cytokines from the local site of injury can be manifested systemically as fever, tachycardia, leucocytosis, and even shock and death.[4]

A tempered and localized cytokine production is beneficial in the postoperative course of patients.

Local IL-1 and TNF-α expression in wounds plays a role in immune response activation, such as neutrophil activation and proliferation of lymphocytes. In addition, these cytokines stimulate wound healing indirectly via fibroblast, immune cell activation, enhanced matrix turnover, and by their direct effects on endothelial cells.[7-10] *In vitro,* in a dose dependent manner, TNF-α administration to endothelial cells causes structural changes and inter-nucleosome cleavage of DNA associated with apoptosis in a concentration and time dependent manner.[11] *In vivo*, TNF-α is a potent pro-angiogenic factor.[9] TNF-α is significantly related to the vascular density index.[12] An *in vitro* study suggested that TNF-α stimulated neutrophils resulting in increased release of intracellular stored vascular endothelial growth factor (VEGF), which is considered as one of the most potent angiogenic growth factors.[13]

IL-6

IL-6 is a pleiotrophic cytokine that can be expressed by various cells. The main sources *in vivo* are stimulated monocytes, fibroblasts, and endothelial cells.

IL-6 is involved locally and systemically in modulation of host immune defense, such as activation of neutrophils and T-cells, and mediating the production of hepatic acute-phase proteins such as C-reactive protein, serum amyloid A, fibrinogen, α1-antitripsin, haptoglobin. In addition, IL 6 induces the expression of vascular endothelial growth factor in a variety of cells.[14-16]

The role of IL-6 in wound healing is still under investigation. Lin et al. conducted a wound study in IL-6-deficient mice and control mice. The IL-6-deficient mice had a delayed closure of the wounded area, with attenuated leukocyte infiltration, re-epithelialization, angiogenesis, and collagen deposition in contrast to the control mice. They suggested that IL-6 played a crucial role in wound healing, probably by regulating leukocyte infiltration, angiogenesis, and collagen accumulation.[17]

Postoperative circulatory IL-6 levels have been found to correlate with the magnitude of the surgical trauma.[16] Numerous studies have demonstrated convincingly lower systemic IL-6 levels after minimal invasive or laparoscopic cholecystectomy than the open procedure.[18,19] In addition, IL-6 may be a predictor of postoperative complications.[18] Pera et al. investigated the influence of postoperative inflammatory responses on angiogenesis and tumor growth. Mice with a coecum tumor were randomized into an open or laparoscopic cecectomy. They suggested that the increased systemic levels of IL-6 and VEGF were associated with increased angiogenesis and tumor growth after open procedure when compared to laparoscopy. In addition, a positive correlation between IL-6 and VEGF postoperative serum levels was found.[20] Of special interest are the IL-6 levels in wound fluid and peritoneal fluid after laparoscopic and conventional surgery, since the source for pro-inflammatory cytokines may be derived from cells accumulated in the wounded area. This may provide insight in wound healing caused by the two different surgical techniques. Several studies have investigated the concentration of pro-inflammatory cytokines locally (wound fluid) and systemically (blood) after laparotomy and mammoplasty and found that the cytokine levels in the wound were markedly higher than in blood. This may indicate compartmentalization, a local accumulation of these cytokines,[21,22] which may be required for local host defense, angiogenesis and wound healing.

IL-8

IL-8 is identified as a neutrophil and T-cell chemotactic factor.[23,24] In addition, IL-8 is found to be a pro-angiogenic factor, inducing proliferation and chemotaxis of human umbilical vein endothelial cells.[25] The fact that IL-8 does not bind directly to ECs suggests that the IL-8 angiogenic effect is indirect.[26] IL-8 is produced by neutrophils, monocytes and T-cells.[27] Non-hematological cells can also generate IL-8, e.g. keratinocytes, fibroblasts and endothelial cells, suggesting a role in wound healing.[28] *In vitro* the effect of recombinant human IL-8 (rhIL-8) resulted in significant keratinocyte proliferation and *in vivo* enhanced re-epithelialization was observed in topically applied IL-8 on human split skin grafts in an experimental model.[29] The postoperative IL-8 response is less frequently studied. Decker et al. demonstrated significantly higher plasma IL-8 level after open cholecystectomy when compared to minimal invasive surgery.[30]

ANGIOGENIC GROWTH FACTORS INVOLVED IN WOUND HEALING

A large number of pro-angiogenic and anti-angiogenic growth factors involved in angiogenesis have been found. Under normal conditions there is a tight physiological control of growth factors through a balance of pro-angiogenic and anti-angiogenic factors. Increased pro-angiogenic factors over anti-angiogenic factors results in angiogenesis. The most important pro- and anti-angiogenic factors, with emphasis on the ones, which have been investigated by us, are discussed here.

PRO-ANGIOGENIC FACTORS

Vascular endothelial growth factor (VEGF)

VEGF is a strong endothelial cell specific mitogen. Five VEGF ligands have been discovered, VEGF-A, VEGF-B, VEGF-C, VEGF-D, and VEGF-E. The most intensively explored is VEGF-A, which includes six isoforms, which are produced due to alternative RNA splicing, and contain 121, 145, 165, 189 and 206 amino acids.[31] $VEGF_{121}$ is completely soluble, whereas $VEGF_{189}$ and $VEGF_{206}$ bind to heparinsulphate glycoproteins (HSGPs) in the extracellular matrix (ECM) and are mainly responsible for VEGF gradients, which direct endothelial cell migration to the sources of VEGF production.

Sofar three high affinity receptors have been identified VEGFR-1 or Fms-like tyrosine kinase (Flt-1), and VEGFR-2 or kinase domain receptor (KDR/Flk-1) are mainly localized on vascular endothelial cells. VEGFR-1 participates in cell migration, while VEGFR-2 is responsible for mitogenic signaling and is considered to be the main regulator of tumor angiogenesis. VEGF-C and VEGF-D are ligands for VEGFR-3, which is expressed on lymphatic endothelial cells in adults. Neuropilin-1 (VEGF-4) binds selectively with VEGF165 and co-ordinates neuronal and vascular development.[31-34]

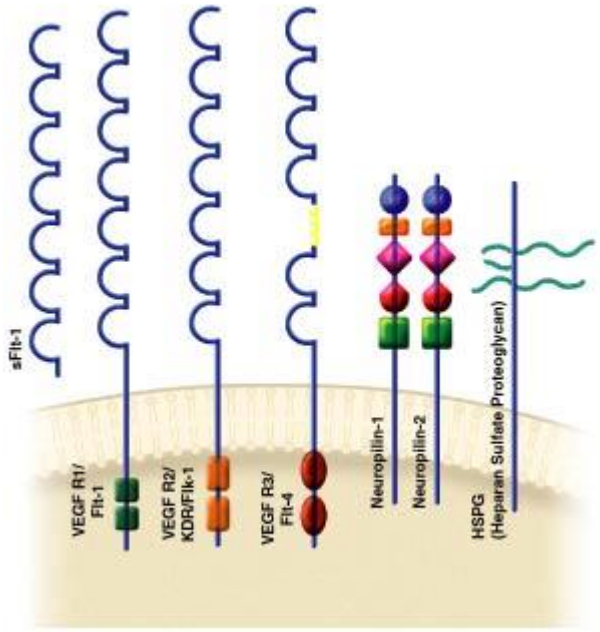

Figure 1. Receptors for VEGF and related ligands include: VEGF R1 (Flt-1), VEGF R2 (KDR/Flk-1), VEGF R3 (Flt-4), Neuropilin-1, and Neuropilin-2. The interaction of heparin-binding forms of VEGF with heparan sulfate may assist in presentation to VEGF receptors. Note: The figure is provided by R&D Systems, Vascular Endothelial Growth Factor (VEGF), in R&D Systems 2000 Catalog.

VEGF production is up-regulated by a wide array of factors, including hypoxia (by stabilization of the transcription factor hypoxia inducible factor 1α (HIF-1α)), hypoglycemia, mutations of oncogenes (Ras, Raf, Src) and suppressor genes (p53), cytokines such as IL-1, IL-6, TNF-α, insulin-like growth factor (IGF-1), transforming growth factor (TGF-α and-β), and basic fibroblast growth factor (bFGF). VEGF is released by a variety of hematological cells, such as platelets, neutrophils, macrophages, T- and B-lymphocytes, as well as non-hematological cells, such as keratinocytes, hepatocytes and almost every type of tumor cells.[31,35-41] Elevated tumor or circulating VEGF levels are predictive of poor survival for many solid tumors, and are associated with enhanced microvessel density in tumor tissue.[42-47] Raised VEGF levels in cancer patients are contributed to tumor cell harboring specific genetic alterations leading to VEGF overproduction. Coagulation abnormalities and increased platelets turnover are also frequently found in patients with cancer. Verheul et al.

demonstrated that the occurrence of platelet adhesion and activation within the tumor may be induced by VEGF activated ECs. This implicates that intratumoral trapping of platelets may be responsible for raised platelet turnover in cancer patients.[48] The discussion whether plasma or serum VEGF levels should be used is still ongoing. Lee et al. suggested that platelets facilitate the process of tumor metastasis by forming aggregates with circulating tumor cells. For this reason, they advocate the usage of serum VEGF in the diagnosis and follow-up of cancer.[49] Coagulation during the processing of serum induces platelet degranulation and subsequent release of stored VEGF.[48] The processing of plasma is not dealing with platelet spillover of VEGF and may therefore represent the actual circulating VEGF concentration, which is about four times lower than in serum.

Raised circulating VEGF levels have been identified after surgery[50,51] which may be generated by local platelet degranulation, recruited leucocytes that are involved in the repair of injured tissue, by cells activated by hypoxia in devitalized tissue and by up-regulation of IL-6 and several growth factors.[16,40,41,52,53] When systemic VEGF levels is compared to local VEGF values a large difference is found. It appears that the circulatory VEGF reflects only a fragment of what is generated in wound fluid.[54]

This observation was supported by Hormbrey et al. who describe the VEGF production in human surgical wounds and the systemic VEGF level changes in patients undergoing benign breast or breast cancer surgery. They suggested that the small blood changes compared with the abundant wound fluid VEGF levels show that there is a tissue barrier.[55]

The local VEGF production is thought to initiate wound angiogenesis, restore the route for oxygen and nutrient delivery and removal of waste products. It induces vascular permeability resulting in deposition of plasma proteins in the wounds or tumor environment. These proteins (fibrin, e.g.) form a provisional matrix, which strongly stimulates the proliferation and migration of endothelial cells. VEGF stimulates granulation tissue formation[56] and has an

anti-apoptotic effect on EC,[57] which may be crucial for survival of ECs during wound healing. In addition, it may be speculated that local VEGF is a physiological immunosuppressive agent in the injured area. Injured tissue contains devitalized tissue and exposed self-antigens that are physiologically controlled by immunosuppressive factors present within the wound.[58] VEGF inhibits the maturation and activation of dendritic cells and may act as an immunosuppressive agent that prevents a local autoimmune reaction. Cancer cells may use the immunosuppressive capacity of VEGF for immunological tumor escape.[59-61]

In summary, the local generation of VEGF is of great importance for wound healing, however VEGF may also have stimulating effects on tumor cells that are left or disseminated in a wound.

Fibroblast growth factor (FGF)

Two forms of FGF, acidic FGF (aFGF or FGF-1) and basic FGF (bFGF or FGF-2) have been identified. This review is restricted to bFGF.

Basic FGF is a growth factor present in normal tissues as well as in tumors. It is a heparin binding polypeptide with several isoforms ranging from 18 to 24 kDa.[62] Basic FGF induces angiogenesis both *in vitro* and *in vivo.*[63,64]

Endothelial cells, granulocytes and platelets contain large amounts of this factor.[62-68] Locally generated bFGF is bound to FGF-receptors in the basement membrane and ECM, where it can be released by ECM-degrading enzymes.[69-70] Wounding as well as degradation of the ECM by invasive tumors may release sequestered bFGF hereby stimulating wound healing, but also tumor growth and angiogenesis.[71,72] The prognostic impact of bFGF is still inconclusive. Several authors demonstrated significant association between tumors expressing bFGF and poor prognosis[72-75] and others did not find such a correlation.[76-78]

ANTI-ANGIOGENIC FACTORS

Several endogenous angiogenic inhibitors have been identified. They may be down regulated during tumor growth or overpowered by the abundance of pro-angiogenic factors. The physiological function of angiogenic inhibitors in wound healing is unclear, but, as it is a physiological process with an initiating and a stopping phase, one might expect that these factors may decrease in the first and reappear in the end phase of wound healing.

Angiostatin

Angiostatin is a 38 kDa internal fragment of plasminogen. Enzymatic digestion of plasminogen by matrix metalloproteinases (MMPs) and plasminogen activators (PAs), such as tissue plasminogen activator (tPA) and urokinase plasminogen activator (uPA), lead to the formation of angiostatin. MMP members such as stromelysin-1 (MMP-3), matrilysin (MMP-7), gelatinase/typeIV collagenase (MMP-9) and macrophage-derived metalloelastase (MMP-12) have been implicated in the *in vitro* production of angiostatin.[79-83] Angiostatin containing kringle 1-5, K1-4 or K1-3 have angiogenesis suppressing activity.[84-86]

O'Reilly et al. demonstrated in an experimental study that a primary tumor could suppress its own micrometastases through production of angiostatin. Surgical excision of that primary tumor removes the production source of angiostatin, which can result in permissive outgrowth of previously dormant micrometastases.[87]

In our study patients undergoing colorectal carcinoma surgery two types of angiostatin (kringle 1-3 and kringle 1-4) became visible postoperatively.[88] The postoperative angiostatin expression is interesting. An *in vitro* study has shown that upon stimulation with a pro-inflammatory stimulus, human PMN release enzymatic activities that generate bioactive angiostatin fragments from plasminogen.[89]

It is also conceivable that the same proteolytic enzymes, involved in early stages of wound healing are involved in the conversion of plasminogen into the angiostatin isoforms.[90] This role of postoperative circulatory angiostatin expression is unclear. It may prevent postoperative vessel sprouting outside the wounded area, by functioning as a systemic homeostatic control, counterbalancing spilled-over circulatory pro-angiogenic factors such as VEGF.

Endostatin

Endostatin is produced from the C-terminal fragment of collagen XVIII by enzymatic digestion. *In vitro* generation of endostatin by elastase and cathepsin L has been demonstrated.[91,92] Collagen XVIII has been shown to reside in basement membranes. Hepatocytes are a major source of collagen XVIII and may contribute to circulating endostatin levels.[93,94] Circulating endostatin values are detectable in both healthy controls as well in cancer patients.[95] It is suggested that endostatin measured in the circulation of healthy volunteers may serve as an angiogenic homeostatic surveillance, controlling undesired vessel outgrowth. Endostatin may temper circulatory VEGF, also detectable in healthy individuals. Hajitou et al. demonstrated in a mouse aortic ring model a down-regulation of VEGF mRNA expression in endostatin-treated rings. A similar down-regulation of VEGF expression at both mRNA and protein levels in tumor cells was also shown in *in vivo* cancer models after treatment with endostatin and angiostatin.[96] Treatment with endostatin decreased also the levels of progenitors of endothelial cells in the circulation *in vivo*.[97] Higher endostatin levels have been detected in some cancer patients,[98,99] suggesting that primary tumors generate proteases able to properly cleave collagen XVIII present in the direct environment.[100] It is unclear whether tumor-derived endostatin, just as angiostatin, is effective in inhibiting its own metastases. The systemic and local endostatin level after a surgical trauma and subsequent

wound healing is interesting. We observed a significant decrease of both systemic and local endostatin levels in patients undergoing benign or cancer surgery.[54,88,101] The precise mechanism of the decrease of endostatin levels is unclear. It may be caused by the expression of various proteolytic enzymes during wound healing resulting in the degradation of endostatin. Alternatively, Wu et al. demonstrated in an *in vitro* study that ECs and pericytes are able to generate endostatin, which decreased under hypoxic conditions. These results suggest that the reduction of autocrine endostatin is an important aspect of hypoxia-driven angiogenesis.[102]

Bloch at al. demonstrated that exogenously administered endostatin impaired blood vessel maturation in mice with excisional wounds on their back.[103]

Treatment with endostatin has shown to inhibit endothelial cell migration *in vitro*, as well as tumor growth in *in vivo* studies.[104-107] The precise mechanisms of endostatins anti-angiogenic activity are complex, with highly interactive angiogenic signaling network (Figure 2).[108] It has been shown that endostatin binds to $\alpha 5\beta 1$ integrin resulting in inhibition of EC migration.[109] Further binding to specific isoforms of tropomyocin in endothelial cells is suggested, leading to a disruption of microfilament integrity and inhibition of cell motility, the promotion of apoptosis by suppressing bcl-2 and the induction of endothelial cell cycle arrest by down-regulating the cyclin D1 promoter *in vitro.*[110-113] A recent study suggested in an *in vitro* study that endostatin predominantly causes autophagic cell death in human endothelial cells through an oxidative-independent pathway, which is regulated by serine and cysteine lysosomal proteases.[114]

Together, the recent evidence concerning the mechanisms of action of endostatin suggests that it brings about an orchestrated anti-angiogenic response by up-regulating a number of essential angiogenesis inhibitors and down-regulating the expression of important angiogenesis stimulators.[108]

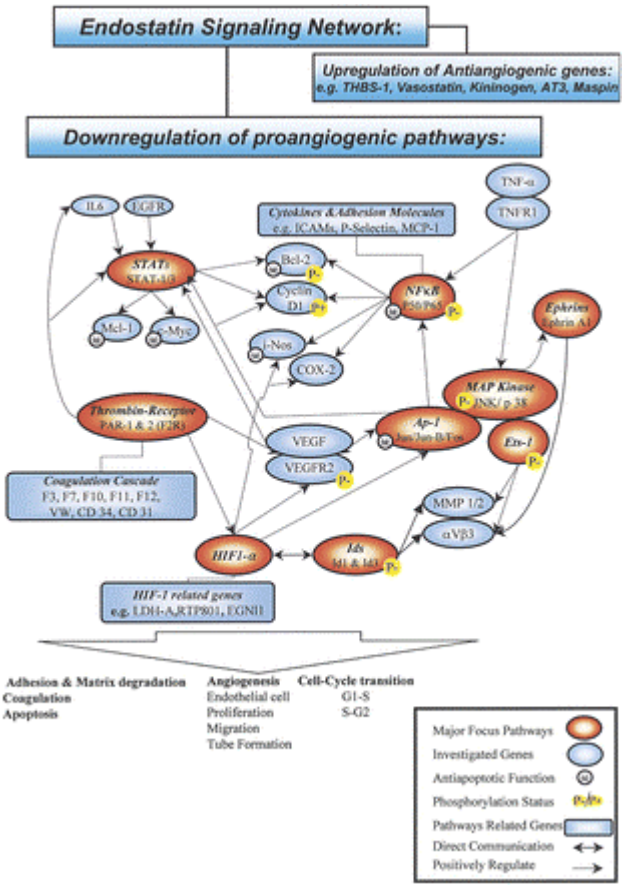

Figure 2. Endostatin Signaling Network
Integration of endostatin signaling network in endothelial cells with emphasis on the downregulation of proangiogenic pathways. Effects of endostatin include the RNA downregulation of key pathways involved in angiogenesis such as Ids, HIF1-α, Ephrins, NF-κB, AP-1 (and MAPK), Stats, Ets, and thrombinreceptors (the coagulation cascade) (orange ovals). The orange ovals represent major pathways that we focused on because of their ability to regulate several genes and their importance for elucidation of the network. Upstream and downstream of these key regulatory elements, endostatin downregulates a cascade of interdependent genes including genes of the VEGF family, Bcl-2, LDH-A, MMPs, TNF-α, COX-2, $\alpha_V\beta_3$ (blue ovals). In addition, endostatin also dephosphorylates many proteins involved in angiogenic cell signaling including Id1, JNK, NF-κB, or Bcl-2 (small circle P-) or phosphorylates proteins such as cyclin D (small circle P+). This network of inter-pathway communications shows that endostatin influences a large number of signaling pathways involved in angiogenesis. [Note the illustration and figure are adapted with permission from A. Abdollahi. Endostatin's Antiangiogenic Signaling Network. Mol Cell. 2004;13:649-63].

WOUND HEALING PROCESS

We mentioned three wound healing phases: hemostasis and inflammation, proliferation and granulation, and scar formation, overlap in time. In these three phases different cells play an eminent role.

PHASE 1: HEMOSTASIS AND INFLAMMATION

Platelets

Injury and the accompanying vessel rupture exposes subendothelial collagen to platelets, resulting in platelet sequestration, degranulation and initiating the clotting cascade. Growth factors such as platelet derived growth factor (PDGF), insulin-like growth factor-I (IGF- I), transforming growth factor (TGF-β), bFGF and VEGF are stored within the α-granules of platelets and are released upon platelet degranulation. Simultaneously, the clotting cascade is initiated consisting of the intrinsic and the extrinsic system. The intrinsic factor is activated by Hageman factor (factor XII) when contact is made between blood and exposed endothelial cell surfaces. The extrinsic factor is initiated by exposure of tissue factor, which is released by tissue damage. The two pathways converge into the final common pathway leading to the formation of fibrin, anaphylatoxins, and the complement factors, C5a and C3a. The formed plug consists of platelets trapped in fibrin fibers, which serve as a temporary cytokine reservoir.[115]

This first wave of cytokines, consisting of growth and chemotactic factors, initiates the wound healing process and recruitment of inflammatory cells.

The degranulation of platelets releasing a first wave of growth factors suggests that platelets are important in the wound healing process. Szpaderska et al. however, could not confirm this idea. They obtained full-thickness excisional dermal wounds from normal and thrombocytopenic mice. The thrombocytopenic mice exhibited no delay in the reparative

aspects of wound healing and the rate of wound re-epithelialization, and collagen synthesis and angiogenesis was nearly identical when compared to control mice. They suggest that platelets do not significantly affect the proliferative aspects of repair, including wound closure, angiogenesis, and collagen synthesis.[116]

Neutrophils

The neutrophils are the first nucleated cell to arrive in minutes to hours by leaving the circulation via endothelial cell transmigration to the site of injury (Figure 3). The migration is promoted by increased vascular permeability and the release of chemotactic substances such as IL-1, IL-6, TGF-β, platelet factor 4 (PF-4) and complement factors. The neutrophils adhere to the endothelium by selectins on the endothelial cell surface.[117] Further migration into the ECM occurs by expressing integrin receptors on the neutrophil cell surfaces.[118]

Activated neutrophils release free oxygen radicals and lysosomal enzyms, which cleanse the injured area from foreign particles. Neutrophils have been mainly considered to be involved in infection control and their contribution to the wound healing process is thought to be minimal.[119] McCourt et al. showed that human neutrophils activated by LPS and TNF-α release VEGF, resulting in stimulation of endothelial cell proliferation and tube formation.[13] In contrary, an *in vitro* study suggested that activated neutrophils by a pro-inflammatory stimuli, generate bioactive angiostatin fragments from purified plasminogen.[89]

A recent study investigated the wound healing process in neutrophil-depleted mice. The epidermal healing, measured by wound closure, proceeded significantly faster in neutropenic than control mice. However, neutrophil depletion did not affect dermal healing, collagen deposition and wound-breaking strength was significantly different between neutropenic and control mice.[120]

In conclusion, neutrophils typically provide a first line defense against infections. In addition, activated neutrophils may also produce a number of growth factors that appear to have stimulatory or inhibitory effect on the angiogenic process.

Macrophages

Macrophages replace the neutrophils in the wound by the third or fourth day. When monocytes leave the vascular system they will adhere to the extracellular matrix and undergo metamorphosis into inflammatory or reparative macrophages. The differentiation of macrophages is mediated by specific cytokines, such as granulocyte-macrophage colony-stimulating factor (GM-CSF), TNF-α, and IL-4.[121]

Reduced vascular perfusion in tissues generates tissue ischemia and a marked reduction in local levels of oxygen and glucose may stimulate macrophages to express pro-angiogenic factors in wounds.[122]

Constant et al. investigated the effects of hypoxia, lactate on the expression of vascular endothelial growth factor by cultured macrophages. A significantly increased level of VEGF mRNA and VEGF protein in the conditioned media was found.[123] The wounds of animals depleted of macrophages healed poorly.[124]

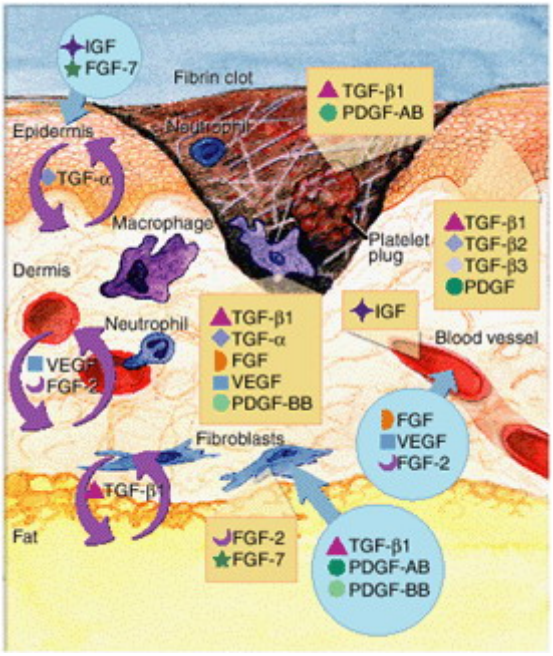

Figure 3. Wound healing is a complex process encompassing a number of overlapping phases, including inflammation, epithelialisation, angiogenesis and matrix deposition. During inflammation, the formation of a blood clot re-establishes hemostasis and provides a provisional matrix for cell migration. Cytokines play an important role in the evolution of granulation tissue through recruitment of inflammatory leukocytes and stimulation of fibroblasts and epithelial cells. [Note the illustrations are provided courtesy of R&D Systems, Inc. Cytokine Bulletin, Winter 2001. The figure is adapted from Singer, A.J. and R.A.F Clark (1999) "Cutaneous Wound Healing" The New England Journal of Medicine 341:738-746].

Epithelialization

Within hours after wounding, the epithelial cells start loosening cell-cell and cell-matrix contacts and migration occurs on the collagen-fibronectin wound surface. Local release of growth factors, loss of contact inhibition and exposure to fibronectin stimulate migration and proliferation until the epidermis reaches its appropriate thickness.

PHASE 2: PROLIFERATION AND GRANULATION

Granulation tissue developing from the connective tissue surrounding the damaged area is a provisional matrix for inflammatory cells, ECs, fibroblasts and myofibroblasts. In the wound platelets and fibroblasts synthesize this provisional matrix. The constituents of granulation tissue include fibrin, fibronectin, and hyalounouric acid. VEGF is a key factor in this process, highlighted by the fact that when neutralizing antibodies experimentally inactivate VEGF, a near complete absence of granulation tissue was observed.[56]

Chemotactic factors and growth factors, such as TGF-β, which are derived from activated macrophages and platelets in the wound, provide a cytokine concentration gradient that coordinates the migration of endothelial cells and fibroblasts. Fibroblasts migrate into the wound site from the surrounding mesodermal elements on the third day after wounding and peak at day seven.[3]

An acellular collagenous matrix gradually replaces the provisional matrix and the production stimulus stops by not well defined signals. Most probably the disappearance of activated cells, the normalization of oxygen tension and the restoration of the angiogenic balance contribute to these stopping signals.

TGF-β has been shown to be the most important growth factor for the differentiation of fibroblasts to contractile wound myofibroblasts,[125] which is required for wound closure.

Angiogenesis

The series of events leading to new vessel growth is complex. Angiogenesis is already initiated minutes after wounding and during wound healing phase tube formation is completed (Figure 4). Local acidosis, hypoxia due to tissue and vessel destruction and local induction of pro-angiogenic factors attribute to the initiation of angiogenesis.

The activation of previously quiescent ECs results in proliferation and migration. Simultaneously, proteolytic enzymes, such as serine proteases (urokinase-type plasminogen activator (uPA) and tissue-type PA (tPA)) and matrix metalloproteinases (MMPs) are released to degrade the ECM allowing EC migration towards the angiogenic source.[126] Angiogenesis ceases once the wound is filled with new granulation tissue and most new vessels will disintegrate as a result of apoptosis.[127] This endothelial program cell death may be regulated by thrombospondin 1 and 2, angiostatin and endostatin and by the decline in VEGF production.[128]

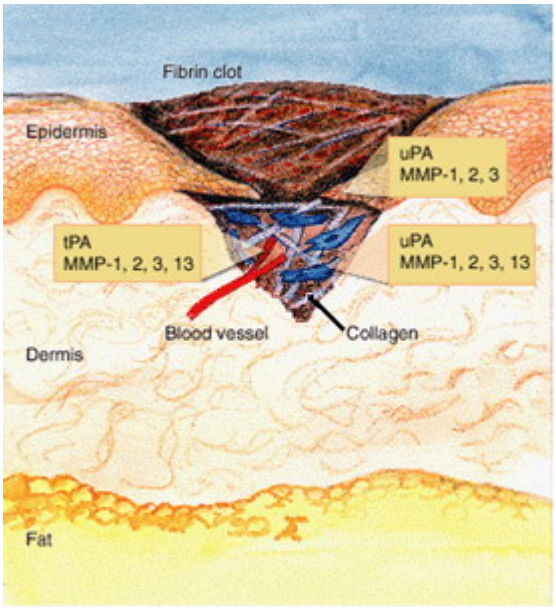

Figure 4. The remodeling phase (*i.e.* re-epithelialization and neovascularization) of wound healing is also cytokine-mediated. Degradation of fibrillar collagen and other matrix proteins is driven by serine proteases and MMPs under the control of the cytokine network. Granulation tissue forms below the epithelium and is composed of inflammatory cells, fibroblasts and newly formed and forming vessels. [Note the illustrations are provided courtesy of R&D Systems, Inc. Cytokine Bulletin, Winter 2001 The figure is adapted from Singer, A.J. and R.A.F Clark (1999) "Cutaneous Wound Healing" The New England Journal of Medicine 341:738-746].

PHASE 3: SCAR TISSUE REMODDELING

Collagen remodeling to scar formation is a dynamic process. Clinically this is the most important phase of healing since the rate, quality and quantity of matrix deposition determines the strength of the scar. Despite the increase in wound strength, the healed wound is at 70% maximum strength when compared to uninjured skin.

WOUND FLUID

Wound fluid is an exudate composed of cell lysate and products secreted by different cells. Wound fluid is believed to reflect the local wound environment and represents the sum of all local specific activities at the time of harvest. Various quantitative cytokine analysis studies in wound fluid, peritoneal fluid and serum indicated that the local cytokine production is much higher than found in the circulation.[129-131] Functional analysis of wound fluid suggested that wound fluid taken in the early healing stages increased the proliferation of fibroblasts and EC, whereas fluid taken from later wound healing phases (day 15) decreased the proliferation of these cells.[132-134] Wound fluid stimulates the synthesis of collagen.[133-134]

Very limited clinical studies have focused on the pro-and anti-angiogenic balance in wound fluid and in the circulation of patients, who underwent surgery because of cancer or other reasons.

SURGERY AND WOUND HEALING

Tumor cells shedded in the circulation during oncologic surgery have been detected[135,136] and are of main concern. Dormant micrometastasis are biologically active with a rate of cell proliferation equal to the rate of apoptosis, with no net growth of the metastasis as a result.[137] The pro-angiogenic environment during wound healing may contribute to the genesis of recurrent disease, locally and at distance.

In 1860 Virchow's studies implied that inflammation during wound healing could function as an initiator of tumor growth. This early concept has been confirmed by current experimental studies that tumors specifically developed in injured tissue and as the wound healed their ability to grow and implant decreased.[138] Bogden et al. demonstrated that surgical wounding of normal tissues significantly stimulated tumor growth at distance.[139] Recently it was demonstrated that pro-angiogenic factors which are generated during wound healing are also involved in the outgrowth of tumors.[140] An immediate increase of circulating VEGF in patients after pulmonary metastasis resection was observed and it has been shown experimentally that VEGF administration resulted in rapid outgrowth of micrometastases. This outgrowth was abolished by an anti-angiogenesis treatment.[51]

In addition, an intact primary tumor can regulate growth of micrometastasis through production of anti-angiogenic factors, notably angiostatin and endostatin. Surgical removal of a primary tumor removes the sources of these inhibitors, and might allow growth of previously dormant micrometastases.[87] This observation has only been demonstrated in animal models but may well exist in humans.

Angiogenesis blocking strategies make use of a wide array of direct, EC targeting, and indirect, influencing the EC microenvironment, anti-angiogenesis agents. Various experimental and clinical anti-angiogenic trials are focused on sustaining perpetual micrometastases in dormancy.[104,141-145] The use of postoperative anti-angiogenic agents in surgical cancer patients is tempting in order to control the excessive production of pro-angiogenic growth factors, which are generated as a physiological response in the early postoperative period. However, these clinical studies are limited, mostly due to an understandable fear of impaired angiogenesis during wound healing.

Roman et al. administered perioperatively the antiangiogenic agent SU5416, an inhibitor of signaling via the VEGFR, in an experimental placebo controlled study in which he performed

a right pulmonary lobectomy and biopsies. Interestingly no gross effect on wound healing in treatment groups was observed. In addition, no drug-related impairment of histologic healing or decrease in wound tensile strength was demonstrated.[146]

Another experimental study evaluated the effect of postoperative continuous or discontinuous angiostatin treatment on the healing of colonic anastomoses. They suggested that the anastomotic healing was impaired when angiostatin was continuously administered, whereas normal colonic healing was restored when the anti-angiogenic agent was preoperative discontinued.[147]

In conclusion, anti-angiogenic therapy combined with surgical treatment may be a future strategy for induction of remission by maintaining tumor cells dormant. However, the timing of anti-angiogenic administration is essential, as wound healing (skin, bowel anastomoses, and liver regeneration) may be impaired by anti-angiogenic agents.

SCOPE OF THE THESIS

This thesis is divided in three parts and addresses the peri-operative angiogenic balance between stimulators and inhibitors of the wound healing process.

PART I

A common pathway in response to trauma is initiated first by interleukin-1 (IL-1) and tumor necrosis factor-α (TNF-α), followed by interleukin-6 (IL-6) and interleukin-8 (IL-8). This consequently recruits immune cells required for host defense and wound healing.

The aims of *Chapter 2* were to study the generation of some important pro-inflammatory cytokines in wound fluid and in the circulation after conventional and minimal invasive surgery in patients with a primary colon carcinoma. In addition, the systemic immune responses after both procedures are investigated.

PART II

Cancers generate VEGF, which may accumulate in their direct environment and increase in the circulation. Therefore, we first wanted to study the effect of surgery on the angiogenic balance in non-cancerous patients in which minimal invasive versus conventional surgery was performed.

The aim of *Chapter 3* was to investigate the effect of the extent of operative trauma of patients without cancer (laparoscopic versus conventional Nissen fundoplication) on the angiogenic balance of VEGF and endostatin in plasma.

In *Chapter 4* we examined the VEGF and endostatin profile in wound fluid and blood of patients undergoing breast surgery. Two patient groups were compared, group I had a breast carcinoma and group II were female-to-male transsexuals undergoing mastectomy.

In *Chapter 5* local and systemic angiogenic changes of VEGF and endostatin in patients undergoing laparoscopic or open surgery for colon cancer were investigated.

PART III

Peri-operative immune therapy is a valuable option to avoid postoperative infections. Various studies have investigated the immunomodulatory response of a wide array of cytokines. These cytokines have anti- (interferon, e.g.) or pro-angiogenic potential, but the effect on angiogenesis has not been studied extensively. RhGM-CSF has been widely used to stimulate the immune system. In addition, it has been suggested that rhGM-CSF affects the process of angiogenesis via multiple pathways. The aim of *Chapter 6* was to investigate the effects of surgery with or without rhGM-CSF on angiogenic parameters, notably VEGF, endostatin and angiostatin, in patients with a colorectal carcinoma.

Perioperative recombinant bactericidal/permeability-increasing protein ($rBPI_{21}$) administration in patients undergoing liver surgery resulted in a reduced incidence of postoperative infectious complications. Recently it was demonstrated that $rBPI_{21}$ had also

anti-angiogenic capacity. *Chapter 7* is a double blind randomized controlled study in which patients with metastasized colorectal carcinoma were enrolled to investigate the effect of liver surgery, with perioperative $rBPI_{21}$ or placebo administration, on circulatory angiogenic cytokines.

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Chapter 2

Systemic and Peritoneal Inflammatory Response After Laparoscopic or Conventional Colon Resection in Cancer Patients: a Prospective, Randomized Trial

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ABSTRACT

Purpose: This study is to evaluate differences in both the peritoneal and systemic immune response following laparoscopic and conventional surgical approaches.

Methods: Patients with a primary carcinoma were prospectively randomized to curative laparoscopic (n = 12) or conventional (n = 14) colon resection. Pro-inflammatory cytokines interleukin-6 (IL-6), interleukin-8 (IL-8) and tumor necrosis factor-α (TNF-α) were measured in the peritoneal drain fluid and in the serum. C-reactive protein (CRP) and leucocyte counts as well as the differences in leucocyte subpopulations and expression of human leucocyte antigen-DR (HLA-DR) on monocytes were measured perioperatively.

Results: Significantly higher pro-inflammatory cytokine levels are found in the peritoneal drain fluid than in the circulation after both procedures. Serum IL-6 and IL-8 levels were significantly lower 2 hours after laparoscopic surgery compared to the conventional procedure. Postoperative cellular immune counts and HLA-DR expression normalized earlier after the laparoscopic approach.

Conclusions: The systemic pro-inflammatory concentrations after both surgical approaches represent only a small fragment of what is generated in the peritoneal drain fluid. Even if the immediate pro-inflammatory cytokines in the serum are significantly lower in the laparoscopic group, the same cytokines locally produced showed no differences, suggesting that both intra-abdominal approaches are equally traumatic. No differences in cellular response between the two groups were observed.

INTRODUCTION

Surgery, whether conventional (CO) or laparoscopic (LP), is a controlled trauma with immunologic consequences. The extent and duration of the postoperative immune suppression depends on the magnitude and type of the intraoperative injury.[1] Postoperative immune suppression may have considerable consequences as it has been related to infectious complications and the development of tumor metastases in animal studies.[2,3] Some clinical research has focused on the prevention or reversal of this immune suppressive state, by modulating the operative trauma or by administration of different growth factors, in order to reduce postoperative morbidity and gain a better prognosis.

In comparing laparoscopic versus conventional surgery, significantly better protection of the systemic immune system was shown with laparoscopic cholecystectomy and Nissen fundoplication than with the conventional approach.[4-6] The differences between laparoscopic and conventional colectomy are less convincing. Laparoscopic resection of colorectal cancer has not gained universal acceptance, because of the fear of port-site metastasis and the fact that the immunologic advantage of laparoscopy remains controversial in clinical trials and limited prospective, randomized trials.[7-12] To understand the differences between these two approaches, systemic but also locally inflammatory and immunologic parameters will add information important to the final clinical outcome.

Circulating pro-inflammatory cytokines such as interleukin-6 (IL-6) and tumor necrosis factor-α (TNF-α) have been related to the extent and severity of the surgical procedure.[13] The main source for these circulating pro-inflammatory cytokines is largely derived from the operative area. Systemic as well as local measurement in peritoneal wound fluid (PDF) of these cytokines may provide insight into the differences of operative trauma here considered. To our knowledge, this has not yet been evaluated in this context.

To assess the preservation of the postoperative immunologic defenses, the expression of human leukocyte antigen-DR (HLA-DR) on monocytes proved to be a reliable parameter,[14] since HLA-DR molecules are a prerequisite for effective antigen presentation and play an important role in the immune response.[14]

In this prospective, randomized study, the systemic and local acute inflammatory responses as well as the immunologic consequences of both surgical procedures have been evaluated. The primary endpoints of the study were to demonstrate differences in both local and systemic immune parameters by evaluating pro-inflammatory cytokines (IL-6, interleukin-8 (IL-8), TNF-α, and C-reactive protein (CRP)), leukocyte counts, and the differences in leukocyte subpopulations and HLA-DR expression after laparoscopic and conventional colon resection for cancer.

STUDY DESIGN

Twenty-six patients were enrolled as part of the international multi-center COLOR (colon cancer laparoscopic or open resection) trial. In this prospective, randomized study, patients were randomly allocated a computer-generated number, which assigned them to undergo either a laparoscopic, or conventional curative colon carcinoma resection. The Ethics Committee of the VU Medical Center, Amsterdam approved this protocol. Informed consent was obtained from all patients. The inclusion and exclusion criteria are according COLOR trial.

Peripheral heparinized plasma and serum plain tube samples (7 ml Vacutainer Systems Europe, Becton Dickinson, from Meylan Cedex France and Plymouth UK, respectively) were collected preoperatively (baseline), 2 hours, 1 day and 4 days after surgery. A low vacuum abdovac drainage system (Astra, Rijswijk, the Netherlands) was left at the resection site for peritoneal fluid drainage. Twenty-four-hour peritoneal fluid production was collected on days 1 and 4. Serum and wound fluid IL-6, IL-8 and TNF-α samples were obtained by centrifugation for 10 minutes at 3,000 rpm at 4^0C. All samples were stored in aliquots at -80^0C until tested.

Phenotyping of Immune Cells

The following immune parameters were determined in fresh (<6h) heparinized venous blood after erythrocyte lysis and paraformaldehyde fixation of whole blood samples (Q-prep, the Coulter Corporation, Miami, FL, USA): the absolute numbers of leucocytes, total lymphocyte count, the counts of monocytes (CD14+), T-helper lymphocytes (CD4+), T-suppressor lymphocytes (CD8+), B-lymphocytes (CD19+), natural killer (NK) cells (CD3neg, CD16/56pos) and the counts of NK-like T-cells (CD3neg, CD16/56pos). In addition, HLA-DR expression on CD14+ cells was evaluated by FACS analysis (FACStar Plus, Becton

Dickinson, San Jose, CA, USA) and expressed as the ratio of fluorescence intensity (mean fluorescence and peak channel) with and without anti-HLA-DR-FITC. All monoclonal antibodies were purchased from Becton Dickinson, San Jose USA and used for overnight staining (each cell pellet with 20 :l 10-fold diluted Moabs or 30-fold diluted anti-HLA-DR, at 4^{o}C in the dark).

Interleukin-6, and -8 and Tumor Necrosis Factor-α

IL-6, IL-8 and TNF-α concentrations in serum and PDF were measured using commercially available enzyme-linked immunosorbent assay kit (Pelikin compact human IL-6, IL-8 and TNF-α ELISA kits, CLB, Amsterdam, the Netherlands).

C-Reactive protein (CRP)

Plasma CRP levels were measured by the immunoturbidimetric method, using the BM/Hitachi 705 (Boeringher, Mannheim, Germany).

STATISTICAL ANALYSIS

The results are reported as mean ± standard error of the mean (SEM). The "Statistical Package for the Social Sciences" (SPSS 7.5 ^{tm}) was used to analyze the data. Overall differences between groups were analyzed by means of a two-way analysis of variance, and if a significant overall difference between groups was found, the two-sample Mann-Whitney U test was used. The Wilcoxon Signed Ranks Test for two related samples analyzed differences within groups. Significance was accepted at a two-tailed $P<0.05$.

RESULTS

The demographic data are shown in Table 1.

The average hospital stay for the laparoscopic group was shorter than in the conventional group, P=0.02. On the first postoperative day after conventional sigmoidectomy, one patient required relaparotomy because of hemorrhaging. One patient who had a laparoscopic right colectomy had a wound abscess that resolved after draining.

	Laparoscopy	Conventional
No. Patients	12	14
Sex (M/F)	2/10	8/6
Age (yr)	66 ± 3	69 ± 2
Astler-Coller (A:B:C)		
Males	0:2:0	0:7:1
Females	0:0:10	0:3:3
Hemicolectomy right	6	8
Sigmoid resection	6	6
Operative time (minutes)	176 ± 15	143 ± 9
Hospital stay (days)	9 ± 0.5#	12 ± 1

Table 1: Demographic data

Mean ± SEM

# $P < 0.05$, laparoscopy versus conventional

Pro-inflammatory cytokines

Serum IL-6, TNF-α, IL-8 and CRP plasma levels were measured in 12 laparoscopic and 14 conventional patients. In addition, the local production of IL-6, IL-8 and TNF-α was evaluated in wound fluid. Tables 2 & 3 summarizes PDF and serum levels of respectively IL-6 and IL-8 matched for five laparoscopic and eleven conventional patients.

Circulating IL-6 and IL-8 are depicted in figures 1 and 2, respectively. In the conventional group, the serum IL-6 level was highest at two hours postoperatively (P=0.01) and was still elevated at day +1 (P=0.001), as compared to baseline values. In the laparoscopic group, increased IL-6 levels were found 2 hours postoperatively (P=0.03) and were highest at day +1 (P=0.01) as compared to baseline values. Between the groups, IL-6 levels were lower in the laparoscopic group than in the conventional procedure (P=0.04) two hours after surgery.

The IL-6 levels were much higher in the PDF than in the serum. Remarkably, serum levels of IL-6 dropped at day +4 despite a continuing increase in local production. In the laparoscopic group, PDF IL-6 concentrations were not significantly lower than in the conventionally operated patients at day +1 and day +4, respectively P=0.257 and P=0.610. In addition, serum IL-6 levels did not differ between the two groups at these time points.

All TNF-α levels in both groups were below the detectable level, < 25 pg/ml in the serum and < 12 pg/ml in the PDF at all time-intervals.

Similar to IL-6 levels, IL-8 serum levels were significantly lower in the laparoscopic group 2 hours after operating (P = 0.03), whereas no differences were found at day +1 and day +4. Local production, expressed as the PDF IL-8 concentration, was much higher than serum IL-8 levels. As shown in Table 3, no differences were found in local IL-8 production between laparoscopic and conventional surgery, although PDF levels P=0.04 tended to be higher in the laparoscopic group at day +4.

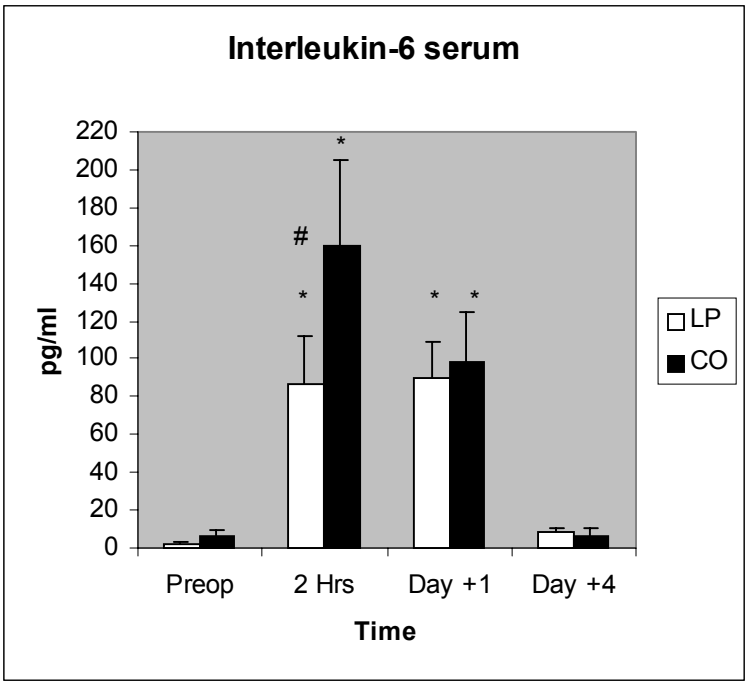

Figure 1: IL-6 levels in mean ± SEM

* P < 0.05, postoperative values compared to baseline levels

# P < 0.05, laparoscopy versus conventional

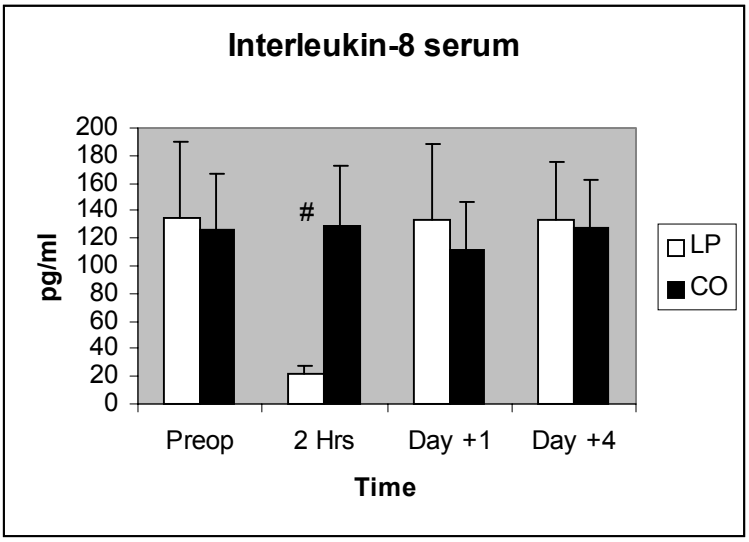

Figure 2: IL-8 levels in mean ± SEM

# P < 0.05, laparoscopy versus conventional

Interleukin-6	Preoperative	Day +1	Day +4
Laparoscopy			
Serum	2.4 ± 0.3	83 ± 7¶	6 ± 1¶
PDF		82.400 ± 28.553	65.600 ± 17.583
Conventional			
Serum	6.4 ± 2.6	105 ± 33¶	17 ± 6¶
PDF		139.909 ± 34.026	80.272 ± 28.277

Table 2: Peritoneal Drain Fluid (PDF)

IL-6 levels in pg/ml

¶ P < 0.05, serum versus PDF

Interleukin-8	Preoperative	Day +1	Day +4
Laparoscopy			
Serum	135 ± 56	67 ± 27¶	134 ± 59¶
PDF		4.209 ± 1.127	17.972 ± 2.385#
Conventional			
Serum	126 ± 41	121 ± 40¶	90 ± 30¶
PDF		7.312 ± 1.511	8.547 ± 2.457

Table 3: Peritoneal Drain Fluid (PDF)

IL-8 levels in pg/ml

¶ P < 0.05, serum versus PDF

# P < 0.05, laparoscopy versus conventional

The CRP plasma levels are depicted in Figure 3. Both the laparoscopic and conventional procedure resulted in increased plasma CRP levels, both with a significant peak increase on day +1 (LP, P=0.003, CO, P=0.001) and on day +4 (LP, P=0.003, CO P=0.001) when compared to baseline values. No differences between the two procedures were observed.

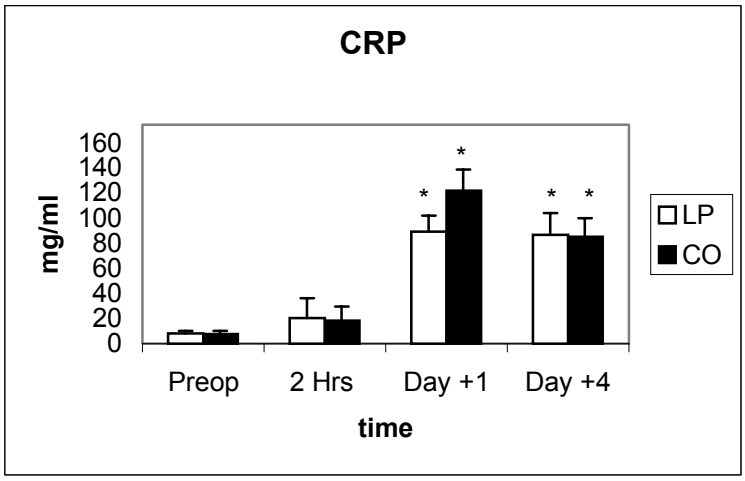

Figure 3: CRP levels in mean ± SEM

* $P < 0.05$, postoperative values compared to baseline levels

Phenotyping of Immune Cells

To assess the cellular inflammatory response of all the twenty-six patients, WBC, lymphocyte subpopulations, NK-Cells and CD14 counts were measured and depicted in Table 4. WBC counts increased on day +1, (LP, P=0.04, CO, P=0.001) in both groups. On day +4, WBC counts normalized after laparoscopy, whereas counts remained elevated in the conventional group (P=0.02).

Total lymphocyte counts dropped on day +1 in both groups, as compared to preoperative values (LP, P=0.007, CO, P=0.006). Again, normalization had begun on day +4 in the laparoscopic group, whereas total lymphocyte numbers remained low in the conventional group (P=0.003) when compared to preoperative levels. The ratio of the lymphocyte

subpopulations expressing CD4 and CD8 was not significantly changed in either the laparoscopic or the conventional group, depicted in Figure 4.

After 2 hours, an initial selective increase of NK-cell (CD3neg, CD16/56pos) counts in both groups was observed. On day +1, a transient decrease in NK counts the laparoscopic group (P=0.01) was observed when compared to preoperative values. In the conventional group, NK-cell counts still showed a decrease on day +4 (P=0.02), whereas NK cells had already normalized by this time in the laparoscopic group.

Increased CD14 counts were already observed 2 hours after laparoscopy and peaked on day +1 (P=0.01); however, in the conventional group, no significant CD14 changes were observed. CD19 counts decreased (P=0.02) in the control group only, observed 2 hours after the procedure.

None of the immune cell parameters showed significant differences between the laparoscopic and conventional procedures.

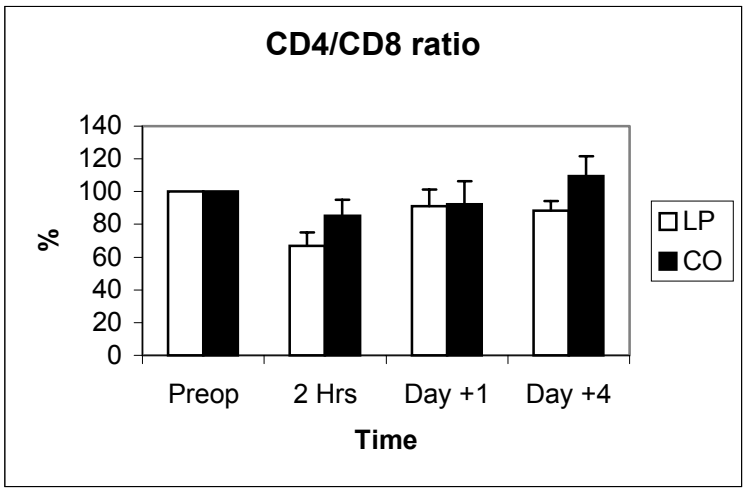

Figure 4: CD4/CD8 ratio in percentage of preoperative value

	Treatment Group	Preoperative	2 Hrs p.o.	Day +1	Day+4
WBC	LP	5.7 ± 0.7	7.1 ± 0.8	8.4 ± 0.6*	6.8 ± 0.7
	CO	6.4 ± 0.6	8.0 ± 0.8	9.9 ± 0.8*	8.3 ± 0.8*
Lymphocytes (total)	LP	1.5 ± 0.2	1.0 ± 0.1	0.8 ± 0.1*	1.1 ± 0.2
	CO	1.4 ± 0.1	1.3 ± 0.2	0.9 ± 0.1*	0.9 ± 0.1*
CD4	LP	761 ± 120	388 ± 38*	351 ± 38*	485 ± 98*
	CO	654 ± 79	458 ± 74*	305 ± 27*	404 ± 47*
CD8	LP	349 ± 44	333 ± 54	197 ± 21*	258 ± 36
	CO	429 ± 54	402 ± 74	253 ± 31*	288 ± 43*
NK-Cells (CD3neg,CD16/56pos)	LP	212 ± 34	263 ± 70	106 ± 13*	240 ± 89
	CO	211 ± 41	272 ± 89	155 ± 23	154 ± 24*
CD19	LP	222 ± 44	128 ± 18	191 ± 39	217 ± 54
	CO	140 ± 21	104 ± 19*	128 ± 25	122 ± 17
CD14	LP	312 ± 44	375 ± 88	589 ± 56*	413 ± 58
	CO	442 ± 53	377 ± 42	535 ± 67	532 ± 55

Table 4: Results are expressed as mean ± SEM

LP = Laparoscopy, CO = Conventional, p.o. = postoperative

White Blood Cell Count (WBC) and lymphocytes in 10^6/ml

CD4+, CD8+, CD14+, CD19+ and NK-cells in 10^3/ml

* $P < 0.05$, postoperative values compared with preoperative levels

HLA-DR Expression

Monocyte HLA-DR expression was used as a parameter for trauma-induced immune suppression and is depicted in Figure 5. The figure shows postoperative HLA-DR expression on monocytes expressed as a percentage of preoperative values set at 100 percent. In both groups, HLA-DR expression decreased within two hours (LP, P=0.01, CO, P=0.02). After laparoscopy, HLA-DR expression was still decreased on day +1, (P=0.05). On day +4 no significant decrease was found, suggesting normalization of the HLA-DR expression. Upon conventional surgery, the HLA-DR expression remained suppressed at least until day +4 (day +1, P=0.002 and day +4, P=0.01).

Significant differences were not observed between the groups.

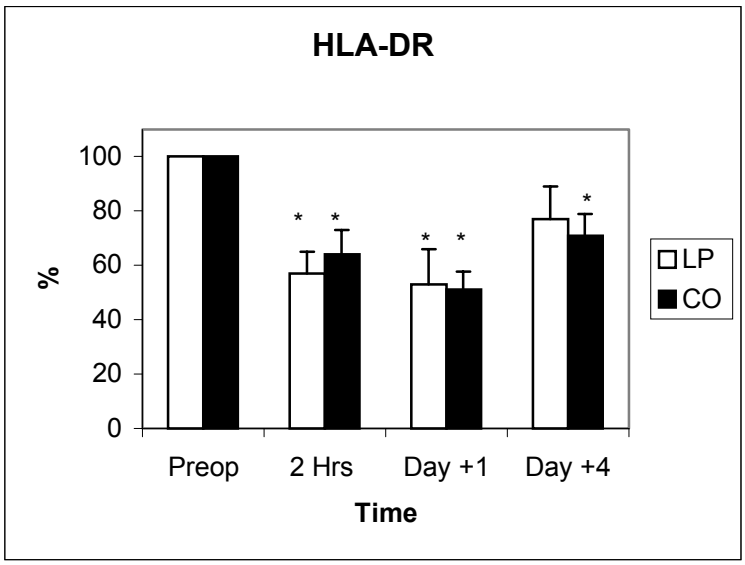

Figure 5: HLA-DR expressions in percentage of preoperative value

* P < 0.05, postoperative values compared to baseline levels

DISCUSSION

In this study, the patients who underwent laparoscopic colon carcinoma resection enjoyed a shorter hospitalization time than patients who underwent conventional surgery. This observation is in accordance with other laparoscopic vs open colorectal resection studies.[15-17] Even if the clinical advantages of laparoscopic approach are clear in procedures such as cholecystectomy or Nissen fundoplication, in which a grade of immunologic preservation is recorded, in more advanced laparoscopic procedures such as colon resection, these advantages become less apparent and sometimes conflictive.

Extensive randomized studies are ongoing in order to demonstrate whether laparoscopic colon resection for cancer is better or worse than or equal in advantages to the conventional approach and to give answers concerning cancer survival. Along with this question, the study of the biologic consequences will offer more insight in the possible advantages of these two different procedures.

The production of the pro-inflammatory cytokines IL-6, TNF-α, and CRP are accurate markers of the overall acute-phase response and cytokine levels in the circulation are used to monitor the impact of surgical trauma.[18] In this study, the serum IL-6 levels taken two hours after surgery were significantly lower after laparoscopic surgery than after conventional surgery and therefore considered less traumatic.

Localized productions of pro-inflammatory cytokines in the PDF after surgical trauma have not been well characterized. The inflammatory cascade is initiated by IL-1 and TNF-α, followed by IL-6[19] and IL-8. Peritoneal mesothelial cells, fibroblasts, macrophages and leucocytes are the probable sources of local cytokine.[20,21] Several studies have reported significant postoperative TNF-α levels measured in wound and peritoneal fluids; however, perioperative circulatory TNF-α changes were undetectable.[12,22,23] In this study, serum and PDF TNF-α levels were undetectable in both groups. This can be explained by the production

of soluble TNF receptors I & II that are shed in the circulation in response to the same stimuli that are known to induce TNF-α. This may compete with other cellular receptors for the binding of free TNF-α.[24]

Serum IL-6 is significantly lower two hours after laparoscopic surgery, suggesting that the laparoscopic approach is initially less traumatic than conventional surgery. In the following days a diminished IL-6 difference between the two groups is in concordance to a previous study of ours.[25]

In both groups, the IL-6 levels in PDF were about a thousand-fold higher than the serum levels. This supports the idea that IL-6 is generated locally and is compartmentalized in response to trauma. Interestingly, no differences were found in PDF on day +1 and +4 between the two groups. This suggests that the local dissected area may be equal after laparocopic and conventional colon surgery. The difference between IL-6 levels in the PDF and serum observed at day +4 is remarkable. IL-6 levels in PDF were still highly elevated whereas the IL-6 levels in serum nearly reached preoperative values at day +4. This observation suggests that circulatory IL-6 is a pivotal factor for the liver to generate CRP; the increased local IL-6 may play a role in local immune cell recruitment and wound healing.

IL-8 is a potent chemoattractant for neutrophil and lymphocyte as well as an angiogenic growth factor promoting wound healing.[26,27] A couple of observations regarding IL-8 levels can be made from this study. Firstly, local IL-8 expression is much higher than levels measured systemically. Finding higher IL-6 and IL-8 expression in the PDF supports the above-mentioned idea of compartmentalization of cytokines within the abdominal cavity. This suggests that IL-6 and IL-8 measured systemically represent only a small fragment of what is generated locally. The precise mechanism of compartmentalization is unclear. It is conceivable that high local cytokines production and/or the disrupted lymph drainage system and vascular continuity, due to surgical trauma, may prevent transfer into the circulation. The

localized production stimulates the influx of immune cells,[28,29] which in turn express cytokines and growth factors beneficial for wound healing and host defense. Secondly, IL-8 PDF levels increase in the postoperative period. The prolonged and increased IL-8 expression in the PDF suggests additional cell sources such as endothelial cells and fibroblasts[30] contributing to the wound healing process. Finally, the significantly lower systemic IL-8 level, two hours postoperatively and higher IL-8 expression in the PDF at day +4 after laparoscopic surgery compared to the conventional approach is unclear. It is tempting to speculate on correlations with pneumoperitoneum, or longer surgical injury and exposure to anesthesia.

It is unclear to what extent the peritoneal fluid drain contributes to the cytokine production; however, it is unlikely that the drain itself causes the thousand folds increase.

CRP is a regulatory component of the innate immune system and participates in tissue repair and regeneration as well.[31] In this study, CRP levels were significantly elevated in the postoperative days following laparoscopic and open colon resection; however, no differences between the groups were found. This was in contrast to our previous study that showed significantly lower CRP levels after laparoscopic Nissen fundoplication compared to the conventional procedure observed at day +1.[6] An explanation for this difference is probably due to trauma size, since Nissen fundoplication is considered less traumatic than colectomy. Both surgical procedures affect the systemic cellular immune numbers and usually a postoperative rise in WBC and a decrease in total lymphocytes are found.[3,10] In this study, normalization in WBC and total lymphocyte levels was achieved earlier after laparoscopy than conventional surgery.

Remarkably, the postoperative CD4/CD8 ratio did not change significantly in either group. Often the proportion of CD4 lymphocyte decrease is greater than that of CD8 lymphocytes and it is suggested that the magnitude of the CD4/CD8 ratio drop is related to the size of the

surgical trauma.[3] The observation of increased CD8 and reduced CD4 activity was more pronounced after conventional cholecystectomy when compared to the laparoscopic approach.[32]

HLA-DR expression on monocytes was measured to assess whether the reduced inflammatory response would be associated with a preservation of postoperative presenting antigen capacity of the monocytes. It has been demonstrated that reduced HLA-DR expression and slow restoration to baseline values after surgery are associated with an increased incidence of infection.[33,34] We have shown previously that HLA-DR expression was better preserved after laparoscopic cholecystectomy than the conventional approach.[35] And in a previous laparoscopic versus conventional study of Nissen fundoplication, we observed that after postoperative HLA-DR decrease in both groups, HLA-DR restoration occurred earlier (within one day) in the laparoscopic group.

In the present study on colectomy, HLA-DR restoration was observed in the laparoscopic group on day +4. The degree of operative trauma may determine the rate of HLA-DR restoration, since colectomy is considered major trauma and Nissen fundoplication moderate. Between the groups we did not find statistical differences in HLA-DR expression at the fourth postoperative day, as in the Ordeman study.[10]

In conclusion, in clinical terms, the patients who underwent laparoscopic surgery enjoyed a shorter hospitalization. Immunologically, large amounts of pro-inflammatory cytokines are produced in the peritoneal drain fluid after both procedures and the systemic responses after both surgical approaches are a small reflection of these local events. In the early postoperative period the pro-inflammatory cytokines levels generated locally suggests that both approaches is equally traumatic. No differences in cellular response between the two groups were observed.

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Chapter 3

The effect of laparoscopic versus conventional Nissen fundoplication on VEGF and endostatin levels in plasma

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ABSTRACT

Background: Vascular endothelial growth factor (VEGF) and endostatin are respectively known as an important endogenous stimulator or inhibitor of angiogenesis. Angiogenesis is essential for wound healing.

The aim of this study was to study the effect of the extent of operative trauma (laparoscopic versus conventional Nissen fundoplication) on the angiogenic balance of VEGF and endostatin in plasma.

Methods: Sixteen patients with gastro-esophageal reflux disease were randomly assigned to undergo either a laparoscopic or a conventional procedure. Postoperative inflammatory response was assessed by measuring white blood cells (WBC) and C-reactive protein (CRP). Plasma VEGF and endostatin levels were monitored by an enzyme-linked immunosorbent assay.

Results: CRP levels one day after surgery were significant lower after laparoscopy compared to conventional surgery (P=0.01). WBC and endostatin were not different in both groups. The VEGF level was decreased two hours after the operation in the laparoscopic group (P=0.04) and returned to preoperative values 24 hours later (P=0.03). In the conventional group, VEGF levels increased four days after surgery (P=0.01).

Conclusion: The extent of the operative trauma influences the VEGF response to surgery. No significant change in endostatin levels was seen.

INTRODUCTION

The formation of new vessel from pre-existing capillary vessels is called angiogenesis. Angiogenesis plays an important role in a variety of processes such as wound healing and tumor growth.[1] Under physiological conditions, adult endothelial cells (ECs) are quiescent and do not proliferate. In response to various stimuli such as trauma and hypoxia, quiescent ECs become activated.

Vascular endothelial growth factor (VEGF) is considered the most potent inducer of angiogenesis, since it stimulates EC proliferation, migration and tube formation.[2] Furthermore, the importance of VEGF enhances vascular permeability which induces local effusion of various plasma proteins and consequently migration of endothelial cells into the injured area.[3] Studies have indicated that VEGF is involved early in the wound healing process.

In response to trauma, platelets aggregate and release growth factors, such as platelet derived growth factor (PDGF), platelet derived-endothelial cell growth factor (PD-ECGF) and VEGF which are contained within the α-granules of platelets.[4,5]

Subsequently, peripheral white blood cells (WBC), such as granulocytes, B and T-lymphocytes and monocytes will shortly appear in the injured area. Each type of cells are also able to release VEGF from their secretory granular compartment.[6,7,8] In addition, pro-inflammatory cytokines, such as interleukin-6 (IL-6), interleukin-1β (IL-1β) and tumor necrosis factor-α (TNF-α) are released locally following trauma. All these cytokines are able to induce VEGF expression.[9-11]

Devitalized peripheral tissue is exposed to hypoxia. Tissue pO_2 is usually low in the centre of the wound, increasing gradually as the wound heals. Under hypoxic in vitro conditions, not only host-derived cells but also tumor cells express VEGF.[12,13,14]

In contrast to VEGF, endostatin is a potent endogenous anti-angiogenic cytokine.

Endostatin specifically inhibits EC proliferation, migration and tube formation in vitro. Furthermore it has the ability to suppress tumor growth.[15]

All together, we suppose that the cellular import and cytokines generated locally will result in a net balance of pro-angiogenic over anti-angiogenic stimuli, which promotes wound healing. It is well accepted that laparoscopic surgery reduces the extent of the surgical trauma in comparison to the conventional technique. It can therefore be speculated that a minor trauma will result in a slighter release of angiogenic factors.

The aim of this randomized study was to compare the influence of laparoscopic and conventional Nissen fundoplication on the production of VEGF, endostatin, and the generation of CRP and white blood cells (WBC), representing the inflammatory response.

PATIENTS AND METHODS

Sixteen patients with gastro-esophageal reflux disease who were randomly assigned as a part of a large national randomized study of laparoscopic versus conventional Nissen fundoplication were included in this study. Demographic data and operation times are shown in Table 1. The operative fundoplication was performed according to the Nissen-Rossetti technique.[16]

None of the patients received heparin or corticosteroids. None were known to suffer from diabetes mellitus, malignancy or cardiovascular disease. The protocol was approved by the institutional review committee of our hospital. Informed consent was obtained from all patients.

Peripheral heparinized blood samples (Vacutainer, Becton Dickinson, Rutherford, NJ, USA) were collected preoperatively (baseline), and 2 hours, 1 day and 4 days after surgery. Plasma samples were processed immediately after collection by centrifuging at 2000 r.p.m. for 10 minutes and then stored at -80°C until processing. Repeated freezing and thawing was avoided.

Clinical data	Conventional (n=8)	Laparoscopic (n=8)
Age (years)	41 ± 13	39 ± 9
Male/Female	5/3	5/3
Surgery Time (min)	105 ± 25	125 ± 18
Hospital Stay (days)	5.5 ± 0.8	5.0

Table 1: Demographic data

The data are presented as mean ± SEM

VEGF

Plasma $VEGF_{165}$ levels were measured using a commercially available enzyme-linked immunosorbent assay (ELISA) kit (R & D system Minneapolis, MN). Optical density was measured at 450 nm using a Spectra Fluor, Tecan plate reader, according to the manufacturer's recommended protocol.

Endostatin

Endostatin was measured using a commercial available enzyme-linked immunosorbent assay (ELISA kit, Cytimmune, College Park, Maryland, U.S.A.). The procedure was according to the manufacturer's recommended protocol.

Pheriperal white blood cell count (WBC)

WBC was measured using a Coulter blood analyzer (Coulter, Bastingstoke, England).

C-reactive protein (CRP)

CRP plasma levels were measured by the immunoturbidimetric method, using the BM/Hitachi 705 (Boeringer, Mannheim, Germany).

STATISTICAL ANALYSIS

The results are reported as mean ± SEM. The "Statistical Package for the Social Sciences" (SPSS 7.5 [tm]) was used to analyze the data. Differences within groups were analyzed by the Wilcoxon Signed Ranks Test for two related samples. Differences between the groups were analyzed by means of a two-way analysis of variance. When significant differences between the two groups were found, the two-sample Mann-Whitney U test was used. Significance was accepted at a two-tailed $P<0.05$.

RESULTS

None of the patients suffered any major complications or needed postoperative blood transfusions; one patient in the conventional group had a wound abcess that resolved after draining. VEGF, endostatin levels are shown in Table 2, WBC counts and CRP levels are shown in Figure 1 and 2, respectively.

	Groups	Preop.	2 Hours	Day 1	Day 4
VEGF	CO	60 ± 23	79 ± 37	53 ± 16	102±21*
	LP	78 ± 29	26 ± 17*	79 ± 22#	55 ± 22
Endostatin	CO	21 ± 4.7	21 ± 3.7	16 ± 3.0	21 ± 3.7
	LP	17 ± 2.2	18 ± 1.5	16 ± 2.9	20 ± 2.5

Table 2: Results Mean ± SEM

CO = Conventional, LP = Laparoscopic

VEGF in pg/ml, Endostatin in ng/ml

* $P < 0.05$, postoperative value compared to preoperative values

# $P < 0.05$, day +1 value compared to 2 hours after the operation

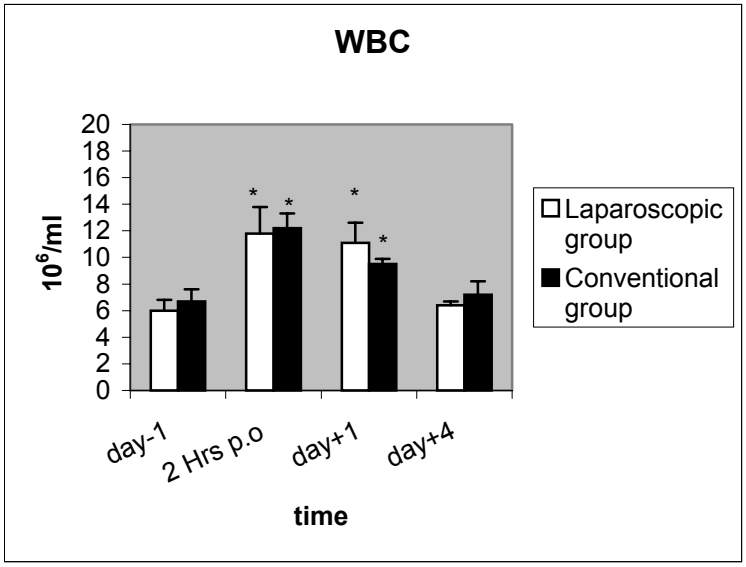

Figure 1: Significant differences in WBC were found postoperatively. In both groups there is an increase of WBC observed 2 hours and day +1.

* P < 0.05, postoperative levels compared to preoperative levels

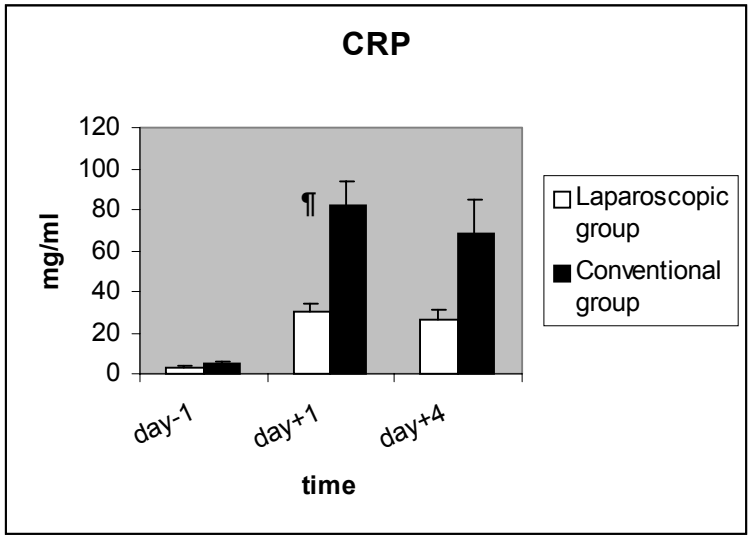

Figure 2: The postoperative inflammatory response is activated on day +1. A significant difference between laparoscopic and conventional surgery were found. After laparoscopic surgery CRP levels increases however not as high as in the conventional group.

¶ P < 0.05, laparoscopic versus conventional surgery

Vascular Endothelial Growth Factor

VEGF levels decreased significantly two hours after the laparoscopic procedure (P=0.04) and returned to the preoperative levels at day+1 (P=0.03).

In the conventional group, VEGF levels increased significantly four days after surgery in comparison with the preoperative values (P=0.01).

No correlation between VEGF and WBC or CRP levels was found.

Endostatin

No significant changes were found in the conventional group and in the laparoscopic group.

White Blood Cell count (WBC)

As expected, WBC significantly increased in the early postoperative period following the laparoscopic and conventional procedure. The same pattern of WBC increase was seen two hours and one day after surgery.

C-Reactive Protein (CRP)

Both surgical procedures resulted in a significant increase in CRP levels on day +1 when compared to the preoperative levels. However, the CRP response was significantly less extensive after laparoscopic surgery compared with the conventional procedure on day +1 (P=0.01). On day +4 the CRP levels in the conventional group were still increased compared to the laparoscopic group.

DISCUSSION

The goal of this study was to evaluate the effect of a minor and major trauma on circulatory VEGF and endostatin levels of patients with a benign disease. The knowledge about the effect of a surgical trauma and subsequent wound healing on the local and systemic angiogenic balance is limited.

The healing of the wound is a complex process and has three consecutive phases, first hemostasis and inflammation (days 0-3 after injury), second the proliferative phase leading to re-epithelialization and granulation formation (days 3-14 after injury) and finally scar tissue remodelling (days 7-30 after injury).[17,18]

The points at which VEGF and endostatin were assessed (preoperative, 2 hours postoperation, day +1 and day +4) allowed us insight in these circulatory angiogenic factor levels in the first two phases of wound healing.

A Nissen fundoplication procedure induces an inflammatory response, which was monitored by means of plasma CRP and WBC. In both groups, WBC increased equally postoperatively. The increase in CRP was more pronounced in the patients undergoing the conventional procedure, as could be expected after major surgery.[19,20]

With respect to VEGF, two significant observations could be made. Firstly, in the laparoscopic group there was a significant decrease in VEGF level two hours after operation, returning to preoperative values on day +1. Secondly, a significant increase in VEGF level was found at day +4 after conventional Nissen procedure. The first observation may be correlated to insufflation of CO_2 for establishing a pneumoperitoneum. Taura et al. demonstrated that CO_2 insufflation at 15 mm Hg caused increased plasma lactic acid which reached its highest value 60 minutes after the operation.[21] Others have confirmed that increased intra-abdominal pressure due to CO_2 insufflation resulted in higher $PaCO_2$ in the postoperative period after laparoscopic approach.[22,23] The decrease of VEGF two hours after

the laparoscopic operation may be explained through direct effects of CO_2 due to insufflation. The increase of VEGF to preoperative level on day +1 may be due to the postoperative effect of acidosis on endothelial cells. D'Arcangelo et al. demonstrated in vitro that hypercarbic acidosis will induces VEGF expression in bovine aortic endothelial cells (BAECs).[24]

The significant increase of VEGF at day +4 only found in the conventional group may be related to the extent of operative trauma. A recent study has shown a transient postoperative increase in serum VEGF levels on the first and third day after major vascular surgery in contrast to minor changes after less extensive surgery.[25]

VEGF is also involved in malignancy.[26] Different tumors are able to produce VEGF and a relationship has been suggested between tumor progression and VEGF levels.[27] Maniwa et al. showed that in patients with lung cancer, the increase of VEGF levels after radical surgery may have a detrimental effect on outgrowth of micrometastases.[28]

In contrast with the pro-angiogenic factor VEGF, endostatin is considered an important anti-angiogenic factor. Endostatin is produced by enzymatic digestion of a protein precursor, collagen XVIII, and has been shown to reside from basement membranes, vessel walls and mostly in the liver.[29,30] The mechanisms of endostatin generation *in vivo* are unknown. Finding endostatin levels in the circulation is interesting and suggests that it may serve as an homeostatic angiogenic control. Theoretically, a net balance of pro-angiogenic over anti-angiogenic factors, would switch in the on position, hereby facilitating wound healing. Nevertheless, in this study the level of endostatin was not influenced by the two surgical techniques.

In conclusion, the levels of VEGF, WBC and CRP change after surgery and the amount of surgical trauma influences the degree of these postoperative changes. The unexpected decrease of VEGF levels immediately after laparoscopic surgery along with unchanged endostatin levels awaits confirmation by additional studies.

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Chapter 4

VEGF and endostatin levels in wound fluid and plasma after breast surgery

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ABSTRACT

Background and Objectives: Angiogenesis is essential for wound repair after surgical trauma. Vascular endothelial growth factor (VEGF) and endostatin are endogenous angiogenic factors involved in the initiation and completion of angiogenesis. The aim of this study was to examine the local and systemic VEGF and endostatin profiles in patients undergoing surgery for benign and malignant breast processes.

Methods: A total of sixteen patients with or without cancer underwent breast surgery. Group I: eight patients with primary breast cancer underwent a simple or radical mastectomy according to Madden including dissection of axillary lymph nodes. Group II: eight healthy female-to-male transsexuals underwent subcutaneous mastectomy. VEGF and endostatin levels in plasma and wound fluid were determined.

Results: In both groups VEGF levels in wound fluid were significantly higher compared to postoperative plasma levels, whereas wound fluid endostatin levels were lower than plasma levels and decreased progressively after surgery. In both groups plasma VEGF and endostatin levels did not change significantly.

Conclusions: The local VEGF increase and endostatin decrease observed immediately after surgery appears to be a physiological response to operative trauma, which can be studied more profoundly in locally generated fluid than in blood. This process did not seem to be influenced by the type of process (cancerous or non-cancerous) involved in the surgical intervention.

INTRODUCTION

Angiogenesis in wound repair is essential to restore the delivery of oxygen and nutrients and the removal of waste products from the injured area. Numerous cells and cytokines are involved during the three phases of wound healing and the balance of stimulatory or inhibitory cytokines will determine each phase of the wound healing process.[1,2] VEGF is a cytokine that is considered the most potent inducer of angiogenesis, since it specifically stimulates endothelial cell (EC) proliferation, migration and tube formation.[3] Furthermore, VEGF enhances vascular permeability, which induces local effusion of various plasma proteins, facilitates the migration of endothelial cells into the injured area[4] and stimulate granular tissue formation.[5] Moreover, in oncology, tumors produce constitutively VEGF as a consequence of their aberrant genetic profile and because of hypoxia inside tumor tissue. The VEGF level is found to correlate with tumor progression.[6,7]

Endostatin, the C-terminal fragment of collagen XVIII, is a potent angiogenesis inhibitor. Hepatocytes are a major source of collagen XVIII and may contribute to endostatin levels in the circulation.[8] Endostatin specifically inhibits EC migration in vitro[9] and potently inhibits tumor growth in various animal models.[10]

In the present study we examined the VEGF and endostatin profile in wound fluid and plasma of patients undergoing curative breast cancer surgery and female-to-male mastectomy. The main interest was to compare the angiogenic profile in wound fluid with the pattern in the circulation.

PATIENTS AND METHODS

The ethical committee of the Vrije Universiteit Medisch Centrum (VUMC) approved the protocol. Informed consent was obtained from all patients.

Group I consisted of eight patients with primary breast ductal carcinomas. A simple mastectomy was carried out on four patients and a drain was left in the resected area. Four other patients underwent a radical mastectomy according to Madden.

Group II consisted of eight healthy female-to-male transsexuals who underwent a mastectomy by a transareolar approach to achieve a male chest figure.

The mastectomies in this group were performed on both breasts and a drain was placed at each side. The wound fluid was collected from the side with the highest wound fluid production. Group II received an androgen supplement (Sustanon® 250 mg i.m., Organon, The Netherlands) twice a month as part of the sex reassignment treatment.

EDTA blood samples were collected preoperatively (baseline), and 1 day and 4 days after surgery. Twenty-four hours of wound fluid production was collected on postoperative days 1 and 4. Plasma and wound fluid samples were centrifuged at 2000 rpm for 10 minutes and then stored at -80°C. Freezing and thawing was avoided.

Detection of VEGF and endostatin

$VEGF_{165}$ and endostatin levels in plasma and wound fluid were measured using a commercially available enzyme-linked immunosorbent assay (ELISA) kit (R & D system Minneapolis, USA). Both intra- and inter-assay variance was less than 10%. The minimum detectable VEGF level is 9.0 pg/ml and endostatin level is 1.95 ng/ml. The local VEGF and endostatin concentration may be affected by a dilution effect of wound fluid production in both groups. Therefore the total VEGF and endostatin production was calculated by

multiplying the concentration by volume for each individual patient, followed by calculating the mean of the levels.

STATISTICAL ANALYSIS

The results are reported as mean ± standard error of the mean (SEM). Overall differences between groups were analyzed by means of a two-way analysis of variance, and if a significant overall difference between groups was found, the two-sample Mann-Whitney U test was used. The Wilcoxon Signed Ranks Test for two related samples analyzed differences within groups. Significance was accepted at a two-tailed $P < 0.05$.

RESULTS

The mean age of the patients included was 51.6 ± 4.9 years for group I and 36.6 ± 4.9 for group II, P=0.04. The average operating time was 128.8 ± 10.9 minutes for group I and 199.3 ± 17.5 minutes for group II, P=0.01. None of the patients experienced any postoperative complication.

The wound fluid production within group I, obtained from the simple mastectomies, (day 1: 70.0 ± 35.6 ml and day 4: 38.8 ± 20.2 ml) and radical Madden mastectomies, (day 1: 90.0 ± 25.8 ml and day 4: 63.8 ± 53.1 ml) was not significantly different.

Four days after surgery the wound fluid production was higher in group I, P=0.001 when compared to group II, displayed in Figure 1.

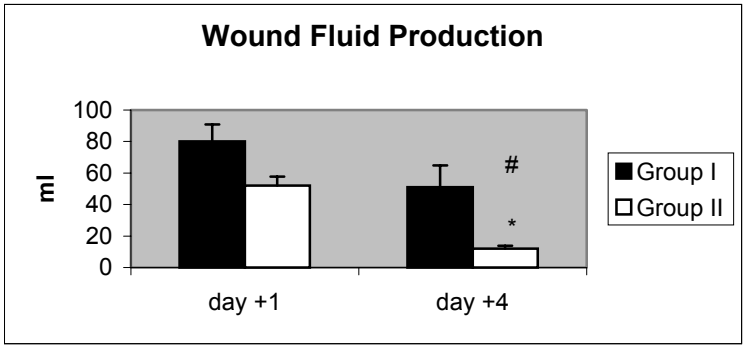

Figure 1: # P < 0.05, differences between the groups

* P < 0.05, day +4 compared to day +1

In both groups, the mean VEGF level in wound fluid was much higher compared to plasma (Table 1). In group I, an eight-fold VEGF increase in wound fluid, P=0.004, compared with plasma level was found one day after surgery. After four days, this difference increased to twenty-four-fold, P=0.018.

In group II, the wound fluid VEGF level increased by eighteen-fold one day after surgery, P=0.001 and thirty-two-fold four days after surgery, P = 0.001, compared to the plasma level of the corresponding day. In both groups, the VEGF concentration in wound fluid was higher at day four compared to day one after surgery, P=0.031. The VEGF levels in plasma and wound fluid were not significantly different between both groups.

In group I, endostatin wound fluid levels were lower than plasma one day, P=0.03, and four days, P=0.004 after surgery. The wound fluid endostatin level decreased after four days compared to day one after surgery, P=0.03. In group II, the endostatin wound fluid level was lower than the plasma level, four days after surgery, P=0.004. In both groups the wound endostatin levels decreased significantly from day one to four days after surgery.

	Prior Surgery	Day +1	Day +4
VEGF Group I:			
Plasma	195.7 ± 42.8	121.5 ± 39.6 ¶	132.2 ± 58.1 ¶
Wound fluid		963.9 ± 187.4	3108.2 ± 703.3 *
Wound fluid (total in pg)		80834 ± 17343	123164 ± 44326
VEGF Group II:			
Plasma	136.9 ± 50.4	62.2 ± 13.6 ¶	95.7 ± 39.7 ¶
Wound fluid		1121.3 ± 120.9	3098.9 ± 488.1 *
Wound fluid (total in pg)		55297 ± 3003	38289 ± 7202
Endostatin Group I:			
Plasma	31.4 ± 2.7	23.1 ± 2.5 ¶	28.5 ± 3.1 ¶
Wound fluid		17.3 ± 1.3	11.8 ± 1.2 *
Wound fluid (total in ng)		1363 ± 215	437 ± 92 *
Endostatin Group II:			
Plasma	23.9 ± 2.3	16.9 ± 1.7	31.7 ± 5.6 ¶
Wound fluid		16.8 ± 1.7	12.7 ± 1.7*
Wound fluid (total in ng)		879 ± 146	162 ± 39 *

Table 1: Mean ± SEM

VEGF in pg/ml, Endostatin in ng/ml

¶ $P < 0.05$, differences between wound fluid and plasma levels

* $P < 0.05$, day +4 compared to day +1

Total = concentration x wound fluid production

DISCUSSION

Angiogenesis plays an important role in the process of wound healing. Insight into the mechanisms of angiogenesis is increasing, but little is known about how local angiogenesis initiated in the wound influences the systemic angiogenic parameters after operative trauma. We evaluated the profiles of VEGF and endostatin in wound fluid and plasma from patients operated for breast cancer or transsexual patients undergoing a double mastectomy.

Wound fluid production in the group with cancer was significantly higher four days after surgery. The fact that their wounded area is larger may explain this.

In this study, the plasma VEGF level was not significantly different between the two groups. It is unknown whether testosterone treatment in women (group II) has an effect on VEGF production.

The systemic VEGF levels after breast surgery did not change significantly, however, the large difference between VEGF in plasma and in wound fluid observed postoperatively in both groups, is remarkable. It appears that the concurrently measured plasma VEGF reflects only a fragment of what is generated locally. Local VEGF generation after a surgical procedure is probably due to instant platelet degranulation, production by recruited immune cells and indirectly by the expression of pro-inflammatory cytokines and local hypoxia due to devitalized tissue.[11-13] In this study it was unlikely that residual tumor was another source of VEGF, since the surgical margins were free of tumor.

The role of a high VEGF level in the wound may have two functions, one to initiate angiogenesis and another to temper the local immune response in the injured area. The devitalized tissue and exposed self-antigens in a wound contain an intense immunologic stimulus. VEGF may act as an immunosuppressive agent[14,15] that prevents a local autoimmune reaction.

In advanced cancer patients, the VEGF level increase may have negative consequences. A recent *in vitro* study showed that breast cancer cells stimulated by VEGF had an increased invasive character.[16] In addition, a VEGF increase induced by surgery resulted in a rapid outgrowth of micrometastases, which could be abolished by an angiogenesis inhibitor.[17] Endostatin levels were also investigated in plasma and wound fluid. Preoperative plasma endostatin levels were not significantly different between the groups and did not change significantly after surgery. However, in contrast to VEGF, endostatin levels decreased in wound fluid in both groups. The mechanism of the decrease of local endostatin levels is unclear. It may be the effect of the expression of various proteolytic enzymes[1,18] during wound healing that degrade endostatin. Anyhow, the decrease of endostatin in the early phase of wound healing seems to be a physiological response to injury. Together, the strong increase in local VEGF and decrease in endostatin supports the idea of a physiological tissue angiogenic switch in the first phase of wound healing. These changes were comparable in both groups, which confirm the idea that wound healing after radical surgery for normal and malignant indications develops through similar physiological mechanisms.

In conclusion, the pattern of change in VEGF and endostatin levels in wound fluid was similar in both patient groups. Wound fluid was much more informative than blood, which emphasizes wound fluid as a "conditioned medium" of this physiological process. In addition, this study showed that angiogenesis is started immediately after surgery. This process did not seem to be influenced by the type of process, cancerous or non-cancerous) involved in the surgical intervention.

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Chapter 5

The systemic and local angiogenic response after laparoscopic or open colon resection in cancer patients: a prospective, randomized trial

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ABSTRACT

Background and Objectives: Angiogenesis is essential for wound healing. Vascular endothelial growth factor and endostatin are both endogenous angiogenic factors, thought to be involved in the initiation, respectively termination of angiogenesis. The aim of this study was to assess the local and systemic angiogenic profile in patients undergoing laparoscopic or open surgery for colon cancer.

Methods: Patients with a primary colon carcinoma were prospectively randomized to curative laparoscopic (n = 12) or conventional (n = 14) resection. Vascular endothelial growth factor and endostatin levels in serum and wound fluid were investigated.

Results: In both groups vascular endothelial growth factor levels in wound fluid were significantly higher compared to postoperative serum levels, whereas endostatin levels in wound fluid were lower than serum levels and decreased progressively after surgery. The vascular endothelial growth factor levels in wound fluid measured at day 4 were significantly higher in the laparoscopy group compared with the laparotomy patients.

Conclusions: Wound healing is associated with a strong local increase in pro-angiogenic factors and a decrease of anti-angiogenic factors. The investigation of locally produced factors offered greater insight in the process of angiogenesis during wound healing than could be acquired from the circulation.

INTRODUCTION

Angiogenesis, the formation of new vessels from pre-existing post-capillary vessels, plays an important role in physiological processes such as healing of wounds and in pathological processes, such as tumor growth and metastases.

Angiogenesis in wound healing is necessary to restore the delivery of oxygen and nutrients and the removal of waste products from the injured area. The healing of the wound is a multi-factorial and complex process involving a cascade of events that overlap in time.[1,2] Numerous cytokines are involved in each phase of wound repair. Among the cytokines involved in wound healing, Vascular endothelial growth factor (VEGF) is considered the most potent inducer of angiogenesis, since it specifically stimulates endothelial cell (EC) proliferation, migration and tube formation.[3] Furthermore, VEGF enhances vascular permeability, which induces local effusion of plasma proteins, facilitating the migration of endothelial cells into the injured area.[4] Multiple cells found in wounds express VEGF,[3] whereas endothelial cells are unique in the expression of VEGF receptors, which suggests that VEGF is necessary for wound healing and granulation tissue formation.[5] In addition, tumor cells produce VEGF and the tissue VEGF level is correlated with increased vessel density and tumor progression.[6]

Endostatin, the C-terminal fragment of collagen XVIII, is a potent angiogenesis inhibitor. Hepatocytes are a major source of collagen XVIII and may contribute to endostatin levels in the circulation.[7] In addition, endostatin levels are elevated in some cancer patients, suggesting that cancer cells are able to generate endostatin.[8] Endostatin specifically inhibits EC migration in vitro[9] and potently inhibits tumor growth in various animal models.[10]

In summary, in the first phase of wound healing a number of activated cells produce proteins locally resulting in a transient, positive balance of angiogenic stimulators over angiogenic inhibitors, which promotes wound healing.

Laparoscopic surgery reduces the extent of the surgical trauma in comparison to the conventional technique. It may therefore be expected that minimal invasive surgery will result in less release of angiogenic factors. In the present study we examined the VEGF and endostatin profile in wound fluid and serum of patients undergoing laparoscopic and conventional colon cancer resection.

STUDY DESIGN

Twenty-six patients were enrolled as part of the international multi-center COLOR (colon cancer laparoscopic or open resection) trial. In this prospective randomized study, patients were randomly allocated a computer-generated number, which assigned them to undergo either a laparoscopic, or conventional curative colon carcinoma resection. The Ethics Committee of the VU Medical Center, Amsterdam approved this protocol. Informed consent was obtained from all patients. Serum plain tube samples (7 ml Vacutainer Systems) were collected preoperatively (baseline), 2 hours, 1 day and 4 days after surgery. A low vacuum abdovac® drainage system (Astra, Rijswijk, the Netherlands) was left at the resection site for peritoneal fluid drainage. Twenty-four-hour peritoneal fluid production was collected on day 1 and 4. Serum and wound fluid samples were obtained by centrifugation for 10 minutes at 3,000 rpm and 4^0C. All samples were stored in aliquots at -80^0C until tested.

Measurement of VEGF and endostatin

$VEGF_{165}$ and endostatin levels in serum and wound fluid were measured using a commercially available enzyme-linked immunosorbent assay (ELISA) kit (R&D system Minneapolis, USA), according to the manufacturer's protocol. Both intra- and inter-assay variance was less than 10%.

STATISTICAL ANALYSIS

The results are reported as mean ± standard error of the mean (SEM). The "Statistical Package for the Social Sciences" (SPSS 7.5 ^{tm}) was used to analyze the data. Overall differences between groups were analyzed by means of a two-way analysis of variance, and significance tested by the two-sample Mann-Whitney U test. The Wilcoxon Signed Ranks Test for two related samples analyzed differences within groups. Significance was accepted at a two-tailed $P < 0.05$.

RESULTS

The average hospital stay for the laparoscopic group was significantly shorter than in the conventional group. On the first postoperative day after conventional sigmoidectomy, one patient required relaparotomy because of bleeding. One patient who underwent a laparoscopic right hemicolectomy had a wound abcess that resolved after draining.

The demographic data

	Laparoscopy	Conventional
No. Patients	12	14
Sex (M/F)	2/10	8/6
Age (yr)	66 ± 3	69 ± 2
Astler-Coller (A:B:C)		
Males	0:2:0	0:7:1
Females	0:0:10	0:3:3
Hemicolectomy right	6	8
Sigmoid resection	6	6
Operative time (minutes)	176 ± 15	143 ± 9
Hospital stay (days)	9 ± 0.5#	12 ± 1

Mean ± SEM

# $P < 0.05$, laparoscopy versus conventional

Peritoneal drain fluid (PDF)

Production of PDF showed no differences between the groups at day 1 (laparoscopic 136 ± 68 ml and open 153 ± 37 ml) and day 4 (laparoscopic 20 ± 5.7 ml and open 32 ± 7.7 ml) after operation respectively.

Angiogenic factors

VEGF and endostatin serum levels were measured in 12 laparoscopic and 14 conventionally treated patients. In addition, local production of both factors was evaluated in wound fluid from 5 laparoscopic and 11 conventional-treatment patients.

In both groups, VEGF levels in serum increased about 2-fold at day 4 compared with baseline values (P =0.001), but with no significant difference between these two groups (Figure 1). In the laparoscopic group, VEGF concentration in PDF was three-fold higher at day 1 compared with serum level at the same day. At day 4 this difference increased to seven-fold (Figure 2). The VEGF increase in PDF was significant at day 4 compared with day 1 (P=0.02). In the conventional group, wound fluid VEGF level was about three-fold higher than serum at day 1 and at day 4 after surgery (P=0.01). The VEGF levels in PDF of the laparoscopic group at day 4 were significantly higher than in PDF from the conventional group (P=0.02).

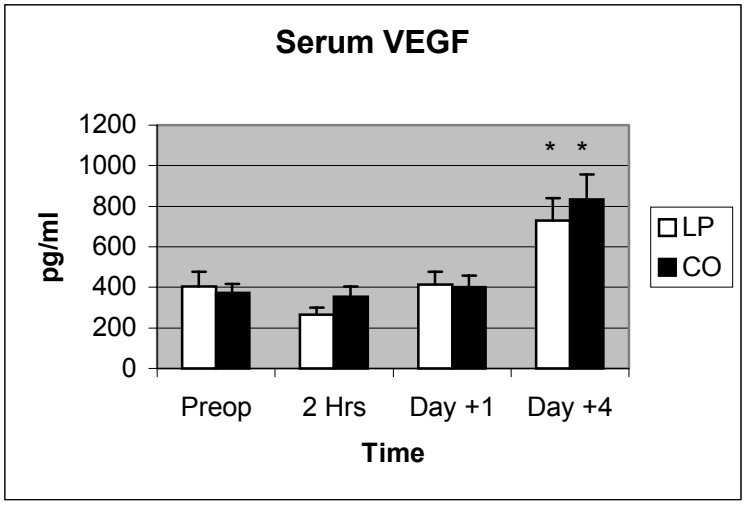

Figure 1: VEGF level in serum

* P < 0.05, day +4 compared to preoperative value

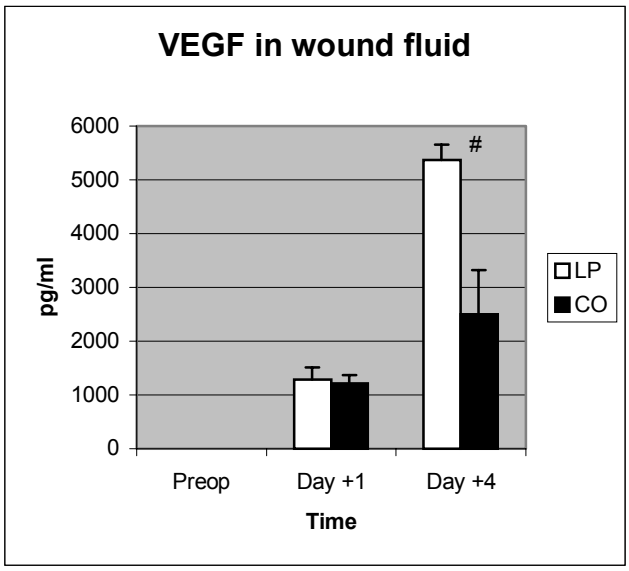

Figure 2: VEGF level in wound fluid

# $P < 0.05$, differences between the groups

Circulating and PDF endostatin levels are depicted in Figures 3 and 4 respectively. In both groups serum endostatin levels were significant lower at day 1 (both, P=0.01) when compared with baseline values. The PDF endostatin levels decreased postoperatively in both groups to concentrations below the detectable level.

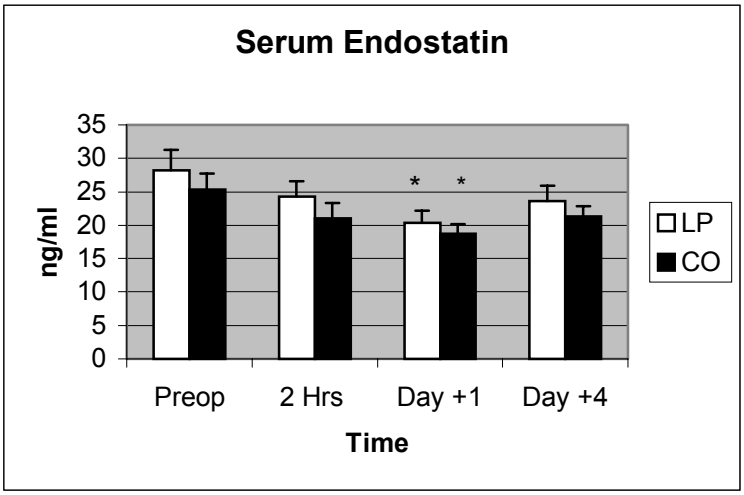

Figure 3: Endostatin level in serum

* P < 0.05, day +4 compared to preoperative value

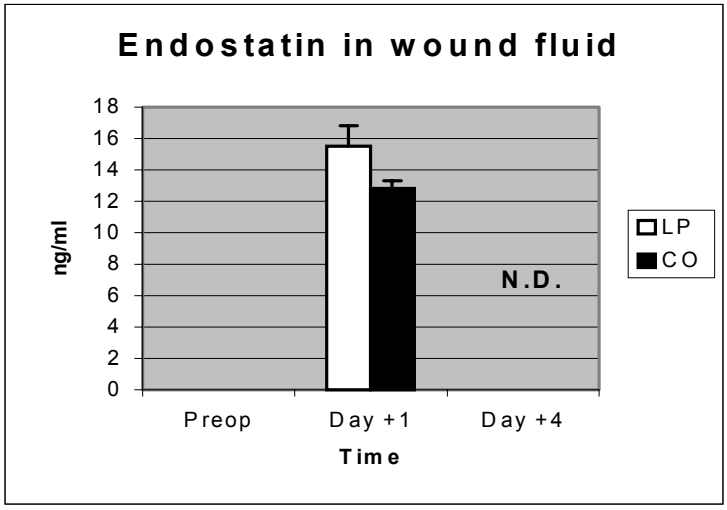

Figure 4: Endostatin level in wound fluid. N.D.= not detectable

DISCUSSION

Clinical and immunologic advantages of laparoscopic approach are clear in procedures such as cholecystectomy or Nissen fundoplication.[11,12] In more extented laparoscopic procedures such as colon resection for cancer these advantages are less apparent.[13] Long term results of a randomized study of Lacy et al. has shown a significant longer survival of stage III colon cancer of patients who underwent laparoscopic surgery when compared to the conventional approach.[14]

We investigated the peritoneal and systemic immune response following laparoscopic and conventional surgical approaches in patients with colorectal carcinoma. We found that large amounts of pro-inflammatory cytokines were produced in the wound fluid after both procedures while the systemic responses after both surgical approaches were only a marginal reflection of these local events. These findings suggest that the surgical wound creates a separate compartment from the systemic circulation in terms of inflammatory response.[13]

We studied two important regulators of angiogenesis at a systemic and a local level after surgery. The data showed increased systemic VEGF levels postoperatively after both conventional and laparoscopic surgery, and significant differences were not seen between both approaches. VEGF levels measured in peritoneal drain fluid were much higher when compared to the systemic values. Local VEGF generation after a surgical procedure is probably due to instant platelet degranulation, production by recruited immune cells and indirectly by the expression of pro-inflammatory cytokines and local hypoxia found in devitalized tissue.[15-17] It was unlikely that residual tumor was another source of VEGF, since the surgical margins were free of tumor. The role of a high VEGF level in the wound may have two functions, one to initiate angiogenesis and another to temper the local immune response in the injured area. The devitalized tissue and exposed self-antigens in a wound contain an intense immunologic stimulus. VEGF may act as an immunosuppressive agent,[18,19]

that prevents a local autoimmune reaction. The significantly higher increase of local VEGF at day 4 in the laparoscopy group versus the laparotomy group is interesting. This may be explained by the postoperative effect of local acidosis, due to CO_2 insufflation, on endothelial cells. D'Arcangelo et al. demonstrated in vitro that hypercarbic acidosis will induces VEGF expression in bovine aortic endothelial cells (BAECs).[20,21]

In contrast to VEGF, the systemic and, more profoundly, the local endostatin levels decreased during the postoperative period. The mechanism of the decrease of local endostatin levels is speculative. It may be due to the release of various proteolytic enzymes during wound healing that degrade endostatin.[1,22] The local decrease of endostatin in the early phase of wound healing may be seen as a physiological response to injury.

In conclusion, the strong increase of local VEGF, and the disappearance of endostatin highlights the concept of a physiological angiogenic switch in the first phase of wound healing. As was shown for immunological parameters,[13] angiogenic factors detected in wound fluid were found at remarkable higher levels when compared to serum after colon surgery. The mechanisms of wound healing can be better studied locally than in the circulation.

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Chapter 6

The effects of surgery, with or without rhGM-CSF, on the angiogenic profile of patients treated for colorectal carcinoma

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ABSTRACT

Wound healing is a process with immunological and angiogenic aspects. RhGM-CSF is known to stimulate the immune system and angiogenesis via multiple pathways. In this study we investigated the combined effects of surgery, with or without rhGM-CSF, on angiogenic parameters in patients with a colorectal carcinoma. In this phase II randomized, placebo-controlled trial, sixteen patients were assigned to perioperative rhGM-CSF (2.8 μg/kg body weight) treatment or saline. Patients received subcutaneous injections from three days before surgery until four days after. IL-6, VEGF, endostatin and angiostatin levels were measured perioperatively. RhGM-CSF enhanced the production of IL-6 and VEGF, but had no effect on the anti-angiogenic agents endostatin and angiostatin. Surgery induced a transient decrease of endostatin. Two types of angiostatin (kringle 1-3 and kringle 1-4) became visible postoperatively. We conclude that this study demonstrated the immediate initiation of angiogenesis postoperatively, reflected by the increase of VEGF and a transient decrease of endostatin, followed by the appearance of two angiostatin bands, which confirms physiological wound healing in these cancer patients.

INTRODUCTION

Radical surgery is the initial treatment for patients with primary colorectal carcinoma. However, major surgery induces immune suppression and tumor resection may induce shedding of tumor cells into the circulation.[1] The transient impairment of the host defense may be associated with bacterial infections and outgrowth of previous dormant micrometastases. RhGM-CSF has been widely used to enhance the number and the activity of granulocytes and monocytes, thereby augmenting host defences.[2]

Apart from immunological activity, GM-CSF also stimulates migration of endothelial cells (ECs) and the formation of new blood vessels from pre-existing vessels, a process known as angiogenesis.[3,4] This process is tightly regulated by pro-angiogenic and anti-angiogenic factors. Among them, vascular endothelial growth factor (VEGF) is considered the most potent and specific pro-angiogenic factor.[5] VEGF production by cells involved in wound healing is partly constitutive, and partly up-regulated by a wide array of external factors, e.g. hypoxia, interleukin-6 (IL-6) and GM-CSF.[6,7] In addition, peripheral blood cells such as platelets and leucocytes contain VEGF, which is released locally after trauma.[8]

Theoretically, GM-CSF might also inhibit angiogenesis by activation of macrophages to produce matrix metalloproteinase-12 (MMP-12). MMP-12 is capable to cleave angiostatin from plasminogen.[9] Angiostatin inhibits endothelial cell proliferation *in vitro*[10] and systemic administration of angiostatin in a murine model induced regression in breast, prostate and colon cancers.[11] MMP-12 produced by macrophages may also be involved in the generation of endostatin.[12] Endostatin induces apoptosis in ECs *in vitro*[13] and potently inhibits tumor growth in animal models.[14]

In short, GM-CSF is pro-angiogenic by stimulating EC directly and enhancing VEGF production. However, GM-CSF may also have anti-angiogenic effects since activated macrophages produce enzymes, which may generate angiostatin and endostatin.

In this randomized study we describe the angiogenic consequences of surgery, with or without perioperative rhGM-CSF administration, in patients with a colorectal carcinoma. The immune stimulating effects of perioperative rhGM-CSF have been published elsewhere.[2]

PATIENTS AND METHODS

Patients with colorectal cancer without detectable metastases underwent curative surgery and were randomized to perioperative treatment with rhGM-CSF or saline. The age of the participants was set for minimum 18 years and maximum 75 years. Patients who received immunosuppressive medication were excluded. No patients were known to have an autoimmune disease. The Ethics Committee of the VU Medical Center, Amsterdam, approved the protocol. Informed consent was obtained from all patients. Sixteen patients were enrolled in the study, of which eight patients were randomly assigned to the rhGM-CSF group and eight patients to the control group. Characteristics of the study population are shown in Table 1.

Table 1: Characteristics of the study population

	Control group	rhGM-CSF group
Gender (male:female)	5 : 3	6 : 2
Mean age (years)	64 ± 4	53 ± 4
Astler-Coller (A:B:C)	0 : 8 : 0	1 : 4 : 3
Males	0 : 5 : 0	0 : 3 : 3
Females	0 : 3 : 0	1 : 1 : 0
Tumor site (Co:S:R)	5 : 2 : 1	3 : 4 : 1
Males	3 : 1 : 1	3 : 2 : 1
Females	2 : 1 : 0	0 : 2 : 0

Age: Mean ± SEM

Astler Coller:

A: tumors with partial wall invasion

B: tumors with transmural involvement

C: regional lymph node involvemen

Tumor site: **Co**: colon, **S:** sigmoid, **R:** rectum

Treatment schedule

All patients were operated through a median laparotomy on day 0. Both groups received daily a subcutaneous injection from 3 days (day –3) before, until 4 days (day +4) after the operation. The patients received either 1 ml of saline or 2.8 µg/kg rhGM-CSF dissolved in 1 ml 0.9% NaCl (Leucomax®, Schering-Plough, Maarssen, The Netherlands).

Samples

Heparinized blood samples were collected on day –4, day 0 (prior to surgery), and day +1 and day +6 after the operation. The cytokine samples were centrifuged at 2000 rpm for 10 minutes and then stored at -80^{0}C.

VEGF

VEGF concentrations were determined in plasma using an enzyme-linked immunosorbent assay (ELISA) kit from R & D system, Minneapolis, U.S.A..

The intra- and inter-assay variance was less than 10%. The range of detection of VEGF was 9.0 - 200 pg/ml.

IL-6

Plasma IL-6 was measured using an ELISA from Pelikine, CLB, Amsterdam, The Netherlands. Normal values are < 10 pg/ml.

Endostatin

Endostatin was measured using an ELISA from Cytimmune, College Park, Maryland, U.S.A.. The intra- and inter-assay variance was less than 10%. The range of detection of endostatin was 1.95 - 500 ng/ml.

Angiostatin

Angiostatin was purified by lysine-sepharose affinity chromatography. Briefly, lysine-sepharose slurry, prepared according to the manufacturer's instructions (Pharmacia, Uppsala, Sweden) was added to plasma samples and incubated under continuous agitation for 15 hours at 4°C. After two washes with 50 mM phosphate buffer pH 6.0, bound material was eluted with 0.2M ε-amino-capronic acid. Aliquots of 15 µl of eluate (equivalent to 60 µl of plasma) plus 15 µl of sample buffer were run on a 10% polyacrylamide gel under non-reducing conditions. After electrophoresis, samples were electroblotted onto nitrocellulose membrane, blocked, and incubated O/N with affinity-purified rabbit anti-plasminogen kringle 1-3 antibody (DAKO, Glostrup, Denmark). This antibody recognizes plasminogen as well as both angiostatin moieties. After washing, blots were incubated for two hours with swine anti-rabbit peroxidase-conjugated secondary antibody (Dako), washed again, and developed by chemiluminescence according to the manufacturer's protocol (Boehringer Mannheim).

STATISTICAL ANALYSIS

Results are reported as mean ± SEM. The "Statistical Package for the Social Sciences" (SPSS 7.5^{tm}) was used to analyze data. Differences within groups were analyzed by the Wilcoxon Signed Rank Test for two related samples. Differences between the groups at different time points were analyzed by means of the two-sample Mann-Whitney test. Significance was accepted at a two-tailed $P < 0.05$.

RESULTS

VEGF, IL-6 and endostatin

The results are shown in Table 2. RhGM-CSF treatment before surgery had no significant effect on these three parameters. One day after surgery VEGF levels were increased in both groups, however more pronounced in the rhGM-CSF group (P = 0.02). In the control group VEGF levels were increased on day +6, (P = 0.01).

IL-6 levels were increased in both groups on day +1 (control, P = 0.01), but in the rhGM-CSF group the increase was more pronounced (P = 0.02).

A significant 3 to 4-fold decrease in the endostatin level was found on day +1 in both groups.

	Treatment Group	**Day - 4**	**Day 0**	Day +1	**Day +6**
VEGF	rhGM-CSF	118 ± 45	218 ± 73	637 ± 59 * #	166 ± 19
	Control	88 ± 28	56 ± 12	160 ± 55	245 ± 91 *
IL-6	rhGM-CSF	11.8 ±16.3	8.8 ± 7.9	116 ± 38.4 *	7.0 ± 2.83
	Control	6.1 ± 0.4	6.0 ± 0.0	41.1± 10.2 *	8.1 ± 5.7
Endostatin	rhGM-CSF	20 ± 2	17 ± 5	4 ± 1 *	17 ± 2
	Control	23 ± 2	18 ± 3	5 ± 1 *	16 ± 3

Table 2: Angiogenic growth factors

Results: Mean ± SEM

VEGF and IL-6 in pg/ml, endostatin in ng/ml

* P < 0.05, postoperative value compared to day -4

# P < 0.05, rhGM-CSF versus control group

Angiostatin

Angiostatin samples were analyzed by Western blot. Examples of Western blots are shown in Figure 1. A prominent plasminogen band at 98 kilo Dalton (kDa), the main source of angiostatin, was present in the plasma of all patients. Next to the plasminogen band, in most samples a doublet of approximately 55 kDa was observed, whereas in some samples a smear between 33-40 kDa with a more discrete band at approximate 37 kDa was present. Based on the specificity of the antiserum used, these bands correspond to angiostatin species consisting of 4 (K1-4) or 3 (K1-3) kringle domains, respectively. The angiostatin data are summarized in Table 3. Whereas a K1-4 angiostatin doublet was absent or vague in most preoperative samples, a clear increase in band intensity at day 6 after operation (Figure 1) was observed in 12 out of 16 patients. Interestingly, the intensity of the K1-4 bands always correlated with the intensity of a protein doublet of high (>116 kDa) molecular weight. The identity of these high molecular weight bands is unknown. K1-3 angiostatin was also not present in the preoperative samples. One day after surgery, however, an intense K1-3 band appeared in 6 out of 16 samples. Treatment with rhGM-CSF had no effect on the angiostatin patterns of these patients.

Patient Number[a]	K1-4 angiostatin (50-55 kDa)				K1-3 angiostatin (33-40 kDa)			
	Day -4	**Day 0**	**Day +1**	**Day +6**	**Day -4**	**Day 0**	**Day +1**	**Day +6**
1 (+)	-	-	-	++	-	-	-	-
2 (+)	-	-	-	++	-	-	++	-
3[b] (+)	-	-	-	++	-	-	+++	-
4 (+)	+	-	-	-	-	-	++	-
5 (+)	n.a.	-	-	+	n.a.	-	-	-
6 (+)	-	-	+	+	-	-	++	-
7 (+)	-	-	-	+++	-	-	-	-
8[b] (+)	+++	+	++	+++	-	-	-	-
9 (-)	-	-	-	-	-	-	-	+++
10 (-)	-	-	-	++	-	-	-	-
11 (-)	-	n.a.	-	++	-	n.a.	+++	-
12[b] (-)	-	-	-	++	-	-	+++	-
13 (-)	-	-	-	++	-	-	-	-
14 (-)	-	-	-	+++	-	-	-	-
15[b] (-)	-	+	-	+	-	-	-	-
16 (-)	-	-	+	+	-	-	-	-

Table 3: Intensity of angiostatin bands in perioperative plasma samples of all colorectal carcinoma patients.

a: Patients: treated with rhGM-CSF (+); control group (-)

b: Patients displayed in figure 1.

K1-4 and K1-3 angiostatin expressions was scored semi-quantitatively. Absent and minimal angiostatin expression were scored (-) to very strong (+++) at various time intervals (days; day 0 prior to surgery).

n.a.: not available

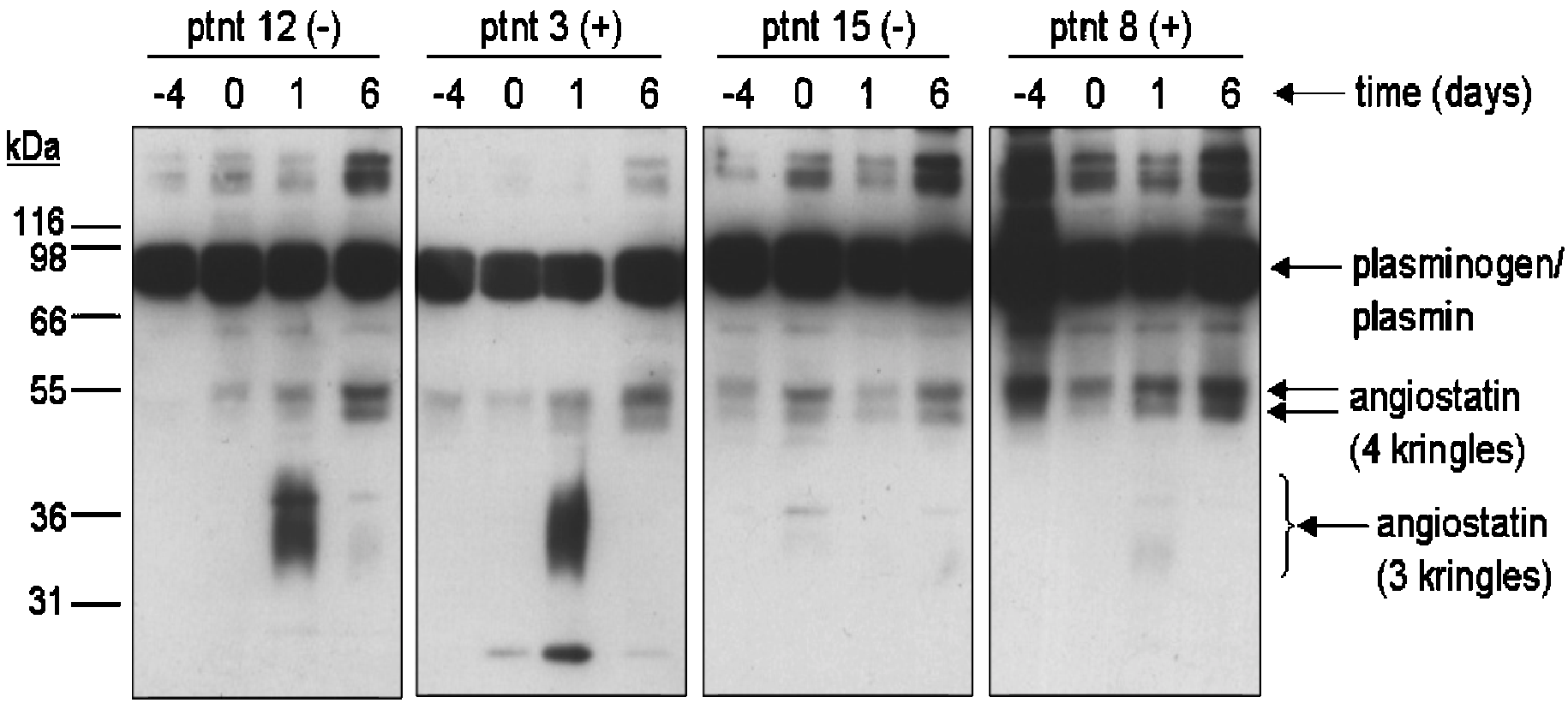

Figure 1: Plasminogen/plasmin, K1-4 and K1-3 angiostatin expressions at various time intervals (days; surgery at day 0) of 4 patients treated with rhGM-CSF (+) or saline (-).

DISCUSSION

Following radical resection for colorectal carcinoma a number of these patients will develop metastatic disease in the liver,[15] which largely determines the overall survival of these patients. Liver metastases may have been already there in an occult form or induced by shedding of cancer cells into the circulation as a result of surgical intervention, and this outgrowth may be stimulated by the effect of postoperative immunosuppression, the angiogenic response as part of wound healing, or the combination of these effects.[1,16] Perioperative rhGM-CSF has been successfully used in order to enhance the immune status of patients with colorectal carcinoma.[2]

In the present study we investigated the effects of surgery, with or without perioperative rhGM-CSF, on VEGF and IL-6 plasma levels. In both groups VEGF increased after surgery, which is in accordance to other observations.[17] Tissue hypoxia is the most probable explanation for the postoperative VEGF increase. A surgical procedure inevitably causes vascular disruption with subsequent reduction of tissue oxygen tension. The observed steep and transient increase of VEGF and IL-6 level in the rhGM-CSF group was remarkable. Two effects may explain the elevated postoperative levels of VEGF induced by rhGM-CSF. First, the surgical trauma itself results in local platelet degranulation and leucocyte sequestration, with local VEGF release. The enhanced number and state of activation of leucocytes induced by rhGM-CSF might have been responsible for the increased VEGF generation. Second, rhGM-CSF induces secondary cytokines, which may affect the VEGF levels.[6,18] The elevated VEGF level found in the rhGM-CSF group would be expected to have a positive effect on wound healing. On the other hand, elevated VEGF levels may have a stimulatory effect on the development of micrometastases, demonstrated in an experimental study.[19] The involvement of VEGF has been further demonstrated in a recent study, where anti-VEGF had a beneficial effect in treatment of metastatic colon cancer.[20]

Furthermore, we investigated whether surgery and rhGM-CSF had an effect on endostatin and angiostatin levels. These are two endogenous anti-angiogenic factors produced by enzymatic digestion of a protein precursor, collagen XVIII and plasminogen, respectively. Collagen XVIII has been shown to reside in tissues such as basement membranes, and in the liver.[21-23] *In vitro* generation of endostatin by elastase and cathepsin L has been demonstrated,[12,23] but the mechanisms of endostatin generation *in vivo* are largely unknown. A recent study suggested that hepatocytes are the major contributors of circulating endostatin. In addition, colon carcinoma may also produce proteases able to properly cleave collagen XVIII present in the direct environment.[24]

In our study, both groups showed a sharp decrease of endostatin immediately after surgery. Various postoperative effects may explain the endostatin decrease. It may concern an effect on the production or clearance of endostatin. Since the half-life of endostatin is less than one hour, an acute reduction of the production of endostatin by hepatocytes as a negative acute phase reaction on stress, or the excision of the tumor could explain an immediate decrease after surgery.

Angiostatin is generated on the endothelial surface from circulating plasminogen. Angiostatin bands appeared in the postoperative period. RhGM-CSF treatment did not affect the fluctuation of angiostatin levels, as these were similar in both the treated and the control group. K1-4 angiostatin became manifest in almost all patients 6 days after surgery, whereas the K1-3 form increased transiently in 6/16 of the patients. Both isoforms may function as a stopping signal for angiogenesis. Cellular migration during early wound healing is dependent on proteolytic enzymes such as matrix metalloproteinases (MMPs) and plasminogen activators.[25] It is conceivable that the same proteolytic enzymes, involved in early stages of wound healing may be responsible for the conversion of plasminogen into the angiostatin isoforms at a latter stage.

In conclusion, we have demonstrated that rhGM-CSF enhanced IL-6 and VEGF. RhGM-CSF had no effect on the production of the anti-angiogenic factors endostatin and angiostatin. However, surgery induced a sharp, but transient decrease of endostatin and a significant increase of angiostatin. The immediate generation of VEGF and decrease of endostatin, followed by the generation of both angiogenesis inhibitors at a later stage of wound healing may reflect a physiological tendency to achieve an angiogenic balance, which is characterized by initiating and stopping angiogenic signals.

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Chapter 7

The effects of major liver resection, with or without recombinant bactericidal/permeability-increasing protein ($rBPI_{21}$), on the angiogenic profile of patients with metastatic colorectal carcinoma

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ABSTRACT

Background: Surgery induces a process of wound healing, which has immunological and angiogenic aspects. Bactericidal/Permeability-Increasing protein (BPI) is found in azurophilic granules of human neutrophils, which is bactericidal and neutralizes lipopolysaccharide (LPS). This may reduce postoperative infectious complications. In addition, BPI has been shown to be an inhibitor of angiogenesis.

Methods: A total of 18 patients with metastasized colorectal carcinoma to the liver were double blind randomized. The levels of the pro-angiogenic factors interleukin-6 (IL-6) and vascular endothelial growth factor (VEGF) and the angiogenesis inhibitor endostatin were investigated after liver surgery with perioperative administration of either $rBPI_{21}$ or placebo.

Results: The highest IL-6 levels were found during the first 24 hours and reached peak levels already at 2 hours postoperatively in both groups. In both groups VEGF levels decreased sharply in the postoperative hours, returning to baseline levels in the days afterwards. In both groups, an immediate decrease in endostatin levels was observed which remained significantly low. $RBPI_{21}$ transiently influenced IL-6 and VEGF.

Conclusions: $RBPI_{21}$ only marginally affected IL-6 and VEGF levels. Surgery per se induced an immediate immune response (IL-6) and an immediate angiogenic response, reflected in an initial VEGF decrease and a longer lasting decrease of endostatin. These findings demonstrate the dynamics of tissue responses in the first phase of wound healing.

INTRODUCTION

Following radical resection for colorectal cancer, still a large number of patients will develop liver metastases,[1] for which major liver resection is a viable option.[2,3] Partial liver resection is a major trauma, and is associated with considerable morbidity such as liver failure, disseminated intravascular coagulation (DIC) and systemic infections.[4,5] We demonstrated earlier that continuous perioperative administration of recombinant bactericidal/permeability-increasing protein ($rBPI_{21}$) administration in patients undergoing partial liver resection resulted in a reduced incidence of postoperative infectious complications.[6] BPI is normally found in azurophilic granules of human neutrophils and has bactericidal properties against gram-negative bacteria and neutralizes lipopolysaccharide (LPS)-mediated effects.[7,8] After liver surgery, the remnant liver has the ability to regenerate to full size. An important factor in the restoration of injured tissue, such as liver regeneration and wound healing is angiogenesis, the formation of new blood vessels. Angiogenesis is regulated by a number of specific stimulators and inhibitors. A recent study showed that BPI also functions as an angiogenic inhibitor.[9] Therefore we were interested in the effects of $rBPI_{21}$ on angiogenic parameters after liver surgery. We measured interleukin-6 (IL-6) and VEGF, since both are potent angiogenic stimulators and known to be involved in liver regeneration, and endostatin, an endogenous angiogenesis inhibitor.

IL-6 is a multifunctional cytokine, which induces VEGF production[10] and is of fundamental importance in liver regeneration. A study by Cressman et al, showed that hepatectomy in IL-6 -/- mice resulted in postoperative liver failure and that IL-6 administration before the hepatectomy normalized hepatocyte proliferation postoperatively.[11]

VEGF is a potent inducer of angiogenesis, since it stimulates endothelial cell (EC) proliferation, migration and vessel formation.[12] An increase in VEGF after surgical procedures has been observed[13,14] and may be directly caused by platelet degranulation or

leucocytes which are involved in the repair of injured tissue, or indirectly by hypoxia in devitalized tissue and by up-regulation of interleukin-6 (IL-6).[10, 13-16] It has been demonstrated in an experimental study that hepatocytes produce VEGF, whereas sinusoidal endothelial cells express the VEGF receptors, flt-1 and KDR/flk-1, suggesting that VEGF may contribute to liver regeneration.[17] Taniguchi et al. showed that postoperative VEGF expression was associated with hepatocyte proliferation and anti-VEGF inhibited hepatocyte proliferative activity after partial hepatectomy in rats.[18]

Endostatin is generated from the C-terminal fragment of collagen XVIII.[19] Endostatin specifically inhibits EC migration *in vitro* and potently inhibits tumor growth in animal models.[20] Endostatin can be generated by elastase and cathepsin L *in vitro*.[21,22] The mechanism of endostatin generation *in vivo* is largely unknown. Collagen XVIII has been shown to reside in basement membranes and vessel walls.[23] A study suggested that hepatocytes are a major source of collagen XVIII and may contribute to endostatin levels in the circulation.[24]

Recombinant BPI ($rBPI_{21}$) is a 21 kDa amino-terminal protein derived from the 55 kDa human BPI_{55}. $RBPI_{21}$ has the same bactericidal properties as the endogenous BPI.[25,26] The main object of this study was to determine the effects of liver surgery on the angiogenic profile in the circulation. In addition, the immunologic and angiogenic effects of $rBPI_{21}$, given in a perioperative infusion were studied.

MATERIALS AND METHODS

Patients. This pilot study was part of a phase II, double-blind, placebo-controlled trial in patients with metastasized colon carcinoma undergoing a partial hepatectomy (PH). The protocol was approved by the Food and Drug Administration, by the Council for Medical Research of the Netherlands Organization for Scientific Research, and by the institutional review boards or ethical review committees of each participating institution. Informed consent was obtained from each patient before entry to this study.

Study design. A patient's eligibility for study entry was based on the predefined criteria listed in Table 1. The patients were assigned to perioperative treatment with $rBPI_{21}$ (8 mg/kg/48hr) or placebo by a 48-hour continuous intravenous infusion starting one hour before liver resection. The $rBPI_{21}$ dose used in this study was based on a previous pharmacokinetic study.[27] Patients were randomly assigned to treatment ($rBPI_{21}$ or placebo) by the central pharmacist at the VU Medical Center. A computer-generated randomization schedule at a 1:1 ratio was used. Patients, investigators and clinical monitors remained blinded to individual treatment until all data were evaluated. Test articles ($rBPI_{21}$ and placebo) were supplied by Xoma US (LLC, Berkeley, California) as a clear, colorless, sterile non-pyrogenic solution in 10 ml glass vials, containing either 2 mg/ml $rBPI_{21}$ or 0.2 mg/ml human serum albumin (placebo) in 5 mM sodium citrate/0.15 M sodium chloride buffer, pH 5.0. The $rBPI_{21}$ solution also contained 0.2 % poloxamer 188 and 0.002 % polysorbate 80. Until use, the $rBPI_{21}$ and placebo solutions were stored at 2-8 ºC. Before the start of the actual liver resection and study-drug infusion, manual exploration and intra-operative ultrasonography were performed to confirm the preoperative findings of tumor size and localization. Only when a resection was estimated to be ≥ 3 liver segments, patients were included and treated with either $rBPI_{21}$ or placebo in an equal volume by 48-hours intravenous infusion. Starting after laparotomy at about 1 hour prior to the actual resection of liver tissue, the study medication was administered into a central vein as the

sole agent during the course of the infusion. The venous access port was never heparinized, but was flushed with physiological saline whenever necessary.

Table 1

Inclusion criteria
Liver metastases from colon carcinoma
Age 18-75 yrs.
Liver resection of ≥ 3 liver segments
Informed consent
Exclusion criteria
Metastases outside the liver
Weight >120 kg
Liver cirrhosis (Child-Pugh class B or C)
Splenomegaly
Traumatic liver injury
Irreversible, fatal underlying disease (including extrahepatic metastases)
Disease or condition causing immunosuppression
Anticoagulant therapy < 48 hr prior to surgery
Immunosuppressive therapy
Lack of commitment to full life support measures or a "Do Not Resuscitate" (DNR) status
Known sensitivity to citrate, albumin, poloxomer or polysorbate
Prior exposure to exogenous BPI

Blood sampling. Blood sampling for the measurements of IL-6, VEGF, and endostatin was performed preoperatively (baseline values) and at postoperative hour 0 (last suture), 2, 4, 8 and postoperative days 1, 3, 5, 7 and 9. Upon collection, blood samples were kept on melting ice, and plasma was obtained by centrifugation for 10 minutes at 1300g at 4°C. All plasma samples were stored in aliquots at -80 ºC until tested.

Assessment of total white blood cells (WBC) and platelets

WBCs including cell differentiation were assessed (Coulter JS, Coulter Electronics, Luton, U.K.). Platelet counts were determined using an automated blood coulter counter. Blood sampling for measurements of WBC and platelets was performed pre- and postoperatively at days 1 and 7.

Assessment of plasma IL-6

The plasma IL-6 concentration was measured using a commercially available enzyme-linked immunosorbent assay (ELISA) kit (Pelikine compact human IL-6 ELISA kit, CLB, Amsterdam, the Netherlands). Normal values are < 10 pg/ml

Assessment of plasma VEGF

$VEGF_{165}$ concentrations were determined using an ELISA kit (R & D system, Minneapolis, USA) according to the manufacturer's guidelines. The intra- and inter-assay variance was less than 10%. The reliable minimum detectable VEGF level is 9.0 pg/ml.

Assesment of plasma endostatin

Endostatin was measured using a commercially available enzyme-linked immunosorbent assay kit (ELISA kit, Cytimmune, College Park, Maryland, USA) according to the manufacturer's guidelines. The intra- and inter-assay variance was less than 10%. The reliable minimum detectable endostatin level is 1.95 ng/ml.

STATISTICAL ANALYSIS

The results are reported as means and standard errors of the mean (SEM). The "Statistical Package for the Social Sciences" (SPSS 7.5 ^{tm}) was used to analyze the data. Overall differences between groups were analyzed by means of a one-way analysis of variance, and if a significant overall difference between groups was found, the two-sample Mann-Whitney U test was used. The Wilcoxon Signed Ranks Test for two related samples analyzed differences within groups. Significance was accepted at a two-tailed $P < 0.05$.

RESULTS

Hemihepatectomy was performed because of metastases of colorectal carcinoma. Of the 31 patients, 8 were not treated because the tumor was either not resectable (5 patients) or could be removed by a small liver resection (3 patients). Five patients were not included due to exclusion criteria. A total of 18 patients were eligible for this study: 9 (6 male/ 3 female) controls and 9 (7 male/2 female) $rBPI_{21}$.

Vascular inflow occlusion by the intermittent Pringle maneuver was used when necessary to reduce blood loss during the transection fase. In the control group, 6 required no vascular occlusion; the other 3 hemihepatectomy had a mean inflow occlusion time of 79 ± 24 minutes. In the $rBPI_{21}$ group, no vascular occlusion was required in 5 patients; the other 4 liver resection patients had a mean occlusion time of 76 ± 38 minutes. In the control group, the mean age was 62.1 ± 2.3 years; the mean operative time was 316.1 ± 26.8 minutes, the mean liver resection was 4.4 ± 0.3 segments, perioperative fluid intake 6977.8 ± 561.2 ml, perioperative blood loss 2694.4 ± 343.2 ml and the mean hospital stay 25.2 ± 5.1 days. In the $rBPI_{21}$, the mean age was 59.4 ± 2.8 years, the mean operative time 317.7 ± 29.8 minutes, the mean liver resection was 4.2 ± 0.2 segments, perioperative fluid intake 7744.4 ± 849.9 ml, perioperative blood loss 3025.0 ± 569.8 ml and the mean hospital stay 19.7 ± 3.1 days. No significant differences were found between the 2 groups. The administration of $rBPI_{21}$ was not associated with any side effect.

The inflammatory response and platelets

Total WBCs, the differential leukocyte and platelet counts of both groups are shown in Table 2. As expected WBC, neutrophils and monocytes significantly increased after surgery in both groups. Lymphocytes and platelets significantly decreased at day +1 in both groups. Treatment with $rBPI_{21}$ had no significant effect on these parameters.

	Time	$RBPI_{21}$	Control
WBC	Baseline	7.8 ± 0.7	6.4 ± 0.6
	Day +1	10.3 ± 0.9*	9.3 ± 0.9*
	Day +7	9.5 ± 0.9	11.6 ± 2.0*
Neutr.	Baseline	5.2 ± 0.5	4.6 ± 0.5
	Day +1	7.6 ± 0.5*	8.5 ± 0.8*
	Day +7	6.9 ± 0.8	7.1 ± 1.6
Mono.	Baseline	0.6 ± 0.09	0.5 ± 0.06
	Day +1	0.7 ± 0.10	0.7 ± 0.07
	Day +7	0.7 ± 0.98	1.0 ± 0.14*
Lymph.	Baseline	1.7 ± 0.2	1.4 ± 0.4
	Day +1	1.0 ± 0.2*	0.8 ± 0.1*
	Day +7	1.1 ± 0.2	1.1 ± 0.2
Platelets	Baseline	264 ± 41	257 ± 19
	Day +1	159 ± 26*	149 ± 10*
	Day +7	210 ± 25	193 ± 21

Table 2: Mean ± SEM

* $P < 0.05$, postoperative values when compared with baseline levels

WBC (White Blood Cells), Neutrophils, Monocytes, Lymphocytes and platelets in 10^6/ml.

Cytokines (Interleukin-6, VEGF, endostatin)

The interleukin-6, VEGF and endostatin concentrations are shown in Table 3 and 4.

Interleukin-6

Plasma IL-6 levels were in the normal range preoperatively, but significantly increased in both groups in all the postoperative periods. In both groups the highest IL-6 levels were found during the first 24 hours and reached peak levels at 2 hours postoperatively. After day +1, the IL-6 levels decreased, though levels remained significantly elevated till day +9 in both groups. The IL-6 levels in the $rBPI_{21}$ group were less increased in all postoperative time intervals. A difference between the two groups was found 2 hours after surgery, P=0.03.

VEGF

In both groups VEGF levels decreased significantly in the postoperative hours, whereas in the control group the VEGF levels even dropped under the minimum detectable levels. At day +1 the VEGF levels increased in both groups. The VEGF level in the $rBPI_{21}$ group was significantly higher than the control group, P=0.01, at day +1.

Endostatin

In both groups, a three to four-fold decrease in endostatin levels was immediately observed, which remained significantly low up to day +3. In the control group the endostatin levels were still low at day +5, (P=0.04) and +7, (P=0.05) when compared to baseline value.

	Time	$rBPI_{21}$	Control
IL-6	Baseline	2.8 ± 1.9	4.2 ± 2.1
	0 Hr	174.9 ± 48.8*	252.8 ± 78.4*
	2 Hr	211.7 ± 52.6* #	527.7 ± 184* #
	4 Hr	181.1 ± 44.8*	486.9 ± 221*
	8 Hr	165.5 ± 36.1*	355.8 ± 161*
	Day +1	168.1 ± 48.9*	133.4 ± 31.3*
	Day +3	45.3 ± 14.0*	55.9 ± 17.7*
	Day +5	38.4 ± 13.0*	70.8 ± 29.2*
	Day +7	25.1 ± 4.9*	48.0 ± 10.8*
	Day +9	20.2 ± 4.4*	43.9 ± 13.7*
VEGF	Baseline	24.3 ± 3.9	35.2 ± 5.5
	0 Hr	17.1 ± 3.8*	N.D.
	2 Hr	10.0 ± 4.1*	N.D.
	4 Hr	14.0 ± 4.9*	N.D.
	8 Hr	9.4 ± 2.1	N.D.
	Day +1	38.4 ± 6.6#	15.6 ± 3.1*#
	Day +3	29.8 ± 5.0	19.9 ± 4.8
	Day +5	24.5 ± 4.5	34.4 ± 10.3
	Day +7	33.3 ± 7.7	27.1 ± 7.9
	Day +9	22.9 ± 4.5	24.0 ± 5.1

Table 3: Mean ± SEM

* $P < 0.05$, postoperative values when compared with baseline levels

# $P < 0.05$, significant difference between the groups

N.D. = not detectable, VEGF and IL-6 in pg/ml

	Time	$rBPI_{21}$	Control
Endostatin	Baseline	33.1 ± 2.9	36.2 ± 3.2
	0 Hr	11.3 ± 1.6*	13.2 ± 1.5*
	2 Hr	14.6 ± 1.6*	15.9 ± 1.3*
	4 Hr	14.2 ± 2.1*	14.3 ± 1.2*
	8 Hr	13.6 ± 2.3*	13.5 ± 1.1*
	Day +1	9.7 ± 1.7*	14.6 ± 1.1*
	Day +3	15.1 ± 1.9*	14.9 ± 2.3*
	Day +5	21.7 ± 1.9	21.1 ± 3.3*
	Day +7	23.3 ± 2.5	17.3 ± 3.1*
	Day +9	24.5 ± 2.8	21.5 ± 4.6

Table 4: Mean ± SEM

* $P < 0.05$, postoperative values when compared with baseline levels

Endostatin in ng/ml

DISCUSSION

In this study, we measured the inflammatory response reflected by the immune cells and IL-6, and the angiogenic response reflected by VEGF and endostatin after partial liver surgery with or without perioperative 48-hour continuous $rBPI_{21}$ administration.

The postoperative increase of total WBC counts was similar in both groups, which is in accordance with other observations.[28] Both groups had elevated postoperative IL-6 concentrations; however, IL-6 levels in the control group were higher than in the $rBPI_{21}$ group, suggesting that $rBPI_{21}$ depresses the IL-6 production. This effect may be explained by the LPS-neutralizing capacity of $rBPI_{21.}$ This observation is in accordance with the study by Meszaros et al. who demonstrated that the release of pro-inflammatory cytokines IL-1 beta, IL-6, IL-8 and tumor necrosis factor, induced by E.coli endotoxin, was tempered by administrating $rBPI_{23.}$[26]

Interestingly, IL-6 levels increased and VEGF levels decreased in the postoperative hours in both groups. This seems paradoxical since IL-6 can induce VEGF[10] and un-physiological as well, since VEGF is needed for the initiation of angiogenesis after surgery. The precise mechanism for this observation is unclear, but the effect may be explained by experimental studies that showed up-regulation of the high affinity VEGF receptors, flt-1 and KDR/flk-1 by the endothelium following partial hepatectomy.[17,29] In the immediate postoperative period, circulatory VEGF and additional VEGF released locally from platelets and leucocytes may be scavenged by the up-regulated VEGF receptors, which is needed for an immediate initiation of the angiogenic process. The data suggest that the remnant liver endothelium acts as a sponge extracting all available VEGF necessary for liver regeneration. Most studies observe an increase of circulating VEGF after surgery but studied this at later time points.[14,30] We demonstrate that profound VEGF physiological dynamics already start in the first postoperative hours.

After 24-hour continuous $rBPI_{21}$ administration, VEGF levels are significantly higher when compared to the control group on day +1. This observation may be explained by the competition of $rBPI_{21}$ with locally generated VEGF on heparin binding sites in the extracellular matrix (ECM), resulting in less storage of VEGF during $rBPI_{21}$ infusion.[9,12] This may alter a VEGF concentration gradient that is required for guided EC migration, confirming the idea of anti-angiogenic effects of $rBPI_{21}$.

The VEGF increase in the later postoperative period may be produced by leucocytes, which play an important role in wound healing, as well as hypoxic cells in devitalized tissue and the increased IL-6 that can induce VEGF expression.[10,16]

Endostatin is an endogenous anti-angiogenic factor. Theoretically, colorectal carcinoma metastasized to the liver can generate endostatin by producing proteases able to cleave collagen XVIII in the direct environment.[31,32] In this study, high preoperative endostatin levels were found in cancer patients. This confirms the hypothesis that colon carcinoma metastasized to the liver may generate endostatin. Both groups showed a significant endostatin decrease immediately after surgery. Multiple factors may be responsible for this effect. Removal of the tumor plus a substantial part of the liver means a reduction of endostatin production. Surgical trauma with subsequent release of various proteolytic enzymes involved in healing of the wound may degrade endostatin. Finally, the effect of anesthesia on the liver may inhibit endostatin production. Since the half-life of endostatin is less than one hour (Entremed Endostatin manual book), these effects together easily explain the immediate decrease after surgery. The postoperative endostatin decrease further substantiates the idea that a tumor may produce angiogenic inhibitors, suppressing their metastases at distance.[33]

In this study, $rBPI_{21}$ only transiently influenced IL-6 and VEGF production. The most striking finding was that surgery per se induced an immediate and impressive immune response (IL-6)

together with an immediate angiogenic response, reflected in a paradoxal VEGF decrease and a longer lasting decrease of endostatin. These immediate responses, even the initial, paradoxal decrease of VEGF, have a clear physiological meaning and emphasize the importance of studying the dynamics of immunological and angiogenic responses in the early phase of wound healing.

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Chapter 8

GENERAL SUMMARY

Since the discovery of angiogenesis by Dr. Folkman in 1971 the explosion of publications demonstrated the great importance biomedical researchers attached to this process for human biology. However, the knowledge about the effect of a surgical trauma on the local and systemic angiogenic balance is limited.

This thesis aimed to evaluate the angiogenic balance in blood and in local fluid in patients:

1. Undergoing elective benign or cancer surgery.
2. Undergoing minimal invasive or conventional surgery.
3. In whom the angiogenic dynamics were measured at early and later time-intervals.
4. Who were perioperatively treated with or without immunomodulators.

This last issue was based on the fact that wounds generate immunologically active cytokines, and that the addition of some specific immunological factors might increase (rhGM-CSF, *chapter 6*) or inhibit ($rBPI_{21}$, *chapter 7*) the immunological response to surgery. This might have consequences for the angiogenic response since many immunological cytokines induce also angiogenic effects.

In *Chapter 2* patients with a primary carcinoma were prospectively randomized to a curative laparoscopic or conventional colon resection. A difference in both the peritoneal and systemic immune response following both surgical approaches was observed. We show evidently that the plasma concentrations of pro-inflammatory cytokines after both surgical approaches represent only a fraction of what is generated locally. Interestingly the appearance of pro-inflammatory cytokines in the circulation is significantly lower in the laparoscopic group, whereas the same cytokines locally produced showed no differences, confirming the idea that both intra-abdominal approaches are different in their impact on the immunological system as a whole. In addition, monocyte HLA-DR-expression was measured as a parameter for trauma-induced immune suppression. A postoperative depression was observed in both groups, but in

the conventional group the HLA-DR-expression remained suppressed at least until four postoperative days. The cellular response in terms of leucocytes was similar between the two groups. Together, subtle differences in the immunological response to a major or minor trauma were observed, which warrants further investigation.

In *Chapter 3* we studied the effect of an operative trauma (minimal invasive versus conventional Nissen fundoplication) on the circulatory angiogenic balance and the inflammatory response. The VEGF plasma level was significantly decreased two hours after the operation in the laparoscopic group and returned to preoperative values after 24 hours. In the conventional group, the VEGF plasma level significantly increased four days after surgery. The white blood cells and C-reactive protein increased rapidly, the latter more intensively in the conventional group. The plasma endostatin concentration remained about the same in both groups. Again, these findings suggest that the extent of a surgical intervention has consequences for the angiogenic and immunological response.

In *Chapter 4* we examined the VEGF and endostatin levels in wound fluid and plasma of patients undergoing curative breast cancer surgery and female-to-male mastectomy. We compared the angiogenic profile in wound fluid with the pattern in the circulation. In both groups VEGF levels in wound fluid were much higher compared to postoperative plasma levels, showing a similar trend as was found for pro-inflammatory cytokines in local fluid and in plasma. The wound fluid endostatin concentration, however, was lower than in plasma and decreased progressively after surgery. The local VEGF increase and endostatin decrease observed immediately after surgery appears to be a physiological response to operative trauma, since endostatin impairs blood vessel maturation during wound healing. This 'angiogenic switch' was not influenced by the type of process (malignant or benign) involved in the surgical intervention.

C*hapter 5* describes the local and systemic angiogenic profile in patients undergoing curative laparoscopic or conventional surgery for colon cancer. Like in *chapter 4* the VEGF levels in wound fluid were significantly higher compared to postoperative circulatory levels, whereas endostatin levels in wound fluid were lower than levels in blood, and decreased progressively after surgery in both groups. Interestingly the VEGF levels in wound fluid measured at day 4 were significantly higher in the laparoscopic group compared with the conventional group. This may be explained by the effect of local acidosis, due to CO_2 insufflation, on endothelial cells.

In the last two chapters the systemic angiogenic balance was evaluated in patients treated with perioperative immunomodulatory agents. Recombinant human GM-CSF is known to stimulate the immune system as well as angiogenesis via multiple pathways. In *chapter 6* we investigated the combined effects of curative surgery, with or without rhGM-CSF, on angiogenic parameters in patients with a colorectal carcinoma. In this phase II randomized, placebo-controlled trial, patients were assigned to perioperative rhGM-CSF (2.8 μg/kg body weight) treatment or saline. RhGM-CSF enhanced the production of IL-6 and VEGF, but had no effect on the anti-angiogenic agents endostatin and angiostatin. Surgery induced a transient decrease of endostatin. After surgery two types of angiostatin (kringle 1-3 and kringle 1-4) became visible in both groups. K1-4 angiostatin became manifest in almost all patients, whereas the K1-3 form increased transiently in 6/16 of the patients. This may be explained by the fact that proteolytic enzymes, which are part of the coagulation pathway and involved in early stages of wound healing, are also active in the conversion of plasminogen into the angiostatin. This study suggests an immediate (within 24 hours) initiation of angiogenesis postoperatively, reflected by the circulatory increase of VEGF and a transient decrease of endostatin, followed by the appearance of two angiostatin bands, which confirms physiological wound healing in these cancer patients.

BPI is bactericidal and neutralizes lipopolysaccharide (LPS), which may reduce postoperative infectious complications. In addition, BPI has been shown to be an inhibitor of angiogenesis. In *chapter 7* patients with metastasized colorectal carcinoma to the liver were double blind randomized. The levels of the pro-angiogenic factors IL-6 and VEGF and the angiogenesis inhibitor endostatin were investigated after liver surgery with a 48-hours during, perioperative administration of either $rBPI_{21}$ or placebo. The highest IL-6 levels were found during the first 24 hours and reached peak levels already at two hours postoperatively in both groups. In both groups VEGF levels decreased sharply in the postoperative hours, returning to baseline levels in the days afterwards. Also, in both groups, an immediate decrease in endostatin levels was observed which remained significantly low. $RBPI_{21}$ only marginally affected IL-6 and VEGF levels. Because blood sampling was performed in the first hours after surgery, it became clear that drastic wounding per se induced an immediate immune response (IL-6) and an immediate angiogenic response, reflected in an initial VEGF decrease and a longer lasting decrease of endostatin. This initial VEGF decrease could be explained by upregulation of the VEGF-receptors as the very first response to wounding, increasing the uptake of available VEGF, followed by an abundant increase in local VEGF production by almost all cells present in the wound and, subsequently, an increase in blood VEGF concentrations.

In conclusion, a surgical trauma elicits a cascade of complex humoral and cellular changes, which is intended to protect the host and promote wound healing. For a wound to heal a temporary dysequilibrium between angiogenic stimulators and inhibitors occurs. In this thesis we demonstrated that a surgical trauma and subsequent wound healing was associated with a strong local increase in angiogenic stimulator (VEGF) and a decrease of angiogenic inhibitor (endostatin), suggesting a sort of angiogenic switch. The fact that we included surgery for patients with a benign or a cancerous process, and the usage of perioperative immunomodulators did not heavily influence this physiological process. Due to the fact that

we studied locally generated fluid together with blood, we could show that both the immunological and the angiogenic consequences of wounding were much more pronounced in wound fluid than in the circulation. This suggests that investigation of wound fluid is much more informative concerning the process of wound healing than the study of blood. Proteomic studies of wound fluid versus blood might be of great interest. In addition, we demonstrated that both the immunological response and the angiogenic response were initiated immediately after wounding, and showed a pattern which is physiological. The inhibitor generated in tissue (endostatin) declined progressively, while enzymatic responses to the formation of an intra-endothelial clot induced two isoforms of a circulating angiogenesis inhibitor (angiostatin).
It may be of great interest to investigate the biological significance of the differences in surgical interventions (minimal invasive or conventional, concerning different organs or areas of the body, different forms of anaesthesia, different types and stages of cancer) for the generation of local and circulatory angiogenic stimulators and inhibitors, and their consequences for tumor biology. An increased local VEGF may support the proliferation of residual tumor cells, and in addition with the immunosuppressive nature of VEGF, which inhibits the maturation of dendritic cells, may ultimately result in permission of tumor cell outgrowth, locally (graft metastases or local recurrence) or at distance. Anti-angiogenic therapy during surgical interventions may have negative consequences for wound healing, but, at the same time, may be beneficial for the tumor status of a cancer patient. Different angiogenesis inhibitors will vary in their effects on wound healing and anti-tumor activity. Timing of these inhibitors during surgery may be of importance. Together, it will be clear that further studies are needed to elucidate the process of angiogenesis during wound healing and the possible effects of angiogenic factors, generated by surgery, on tumor biology.

SAMENVATTING

Angiogenese is het proces van de aanmaak van nieuwe bloedvaten. Dit vindt met name plaats in een embryo, maar ook tijdens de menstruatie en wondgenezing en bij ziektes, zoals kanker. Angiogenese is ontdekt door Dr. Folkman in 1971 en daarna ontstond een explosie van publicaties over dit proces. De kennis van het effect van chirurgisch trauma op angiogenese is echter beperkt. Het doel van dit onderzoek was het angiogene evenwicht in bloed en in wondvocht van patiënten te evalueren. De patiënten in de onderzoeksgroep voldeden aan èèn van de volgende kenmerken:

1. Patiënt heeft goedaardige of kanker chirurgie ondergaan
2. Patiënt heeft minimaal invasieve of conventionele chirurgie ondergaan
3. Bij de patiënt is de angiogene dynamiek gemeten, direct na de ingreep en op latere tijdstippen na de ingreep
4. Patiënt is perioperatief behandeld met of zonder immunomodulators.

De aandacht voor het verschil in perioperatieve behandeling is gebaseerd op het feit dat wonden cytokinen genereren en dat de toevoeging van sommige specifieke immunologische factoren de postoperatieve immunologische respons zou kunnen stimuleren (rhGM-CSF, *hoofdstuk 6*) of remmen (rBPI21, *hoofdstuk 7*). Dit heeft mogelijk gevolgen voor de angiogene respons, want het is bekend dat vele immunologische cytokinen ook angiogene effecten hebben.

In *hoofdstuk 2* werden patiënten met darmkanker zonder uitzaaiing prospectief gerandomiseerd naar een laparoscopische of conventionele ingreep. Een verschil in zowel de lokale (wondvocht) als systemische (plasma) immuunreactie die op beide chirurgische benaderingen volgt, werd opgemerkt. Er wordt aangetoond dat de pro-inflammatoire cytokine concentraties gemeten in plasma na beide chirurgische benaderingen slechts een fractie zijn van wat er plaatselijk gegenereerd wordt. Opmerkelijk is dat de gemeten expressie van pro-inflammatoire cytokinen in plasma beduidend lager was na een laparoscopische ingreep,

terwijl dezelfde expressie gemeten in wondvocht geen verschillen toonde. Hiermee bevestigen we het idee dat beide intra-abdominale benaderingen verschillend zijn in hun uitwerking op het immunologische systeem als geheel. Tevens werd HLA-DR-expressie op monocyten gemeten, wat als een erkende parameter voor postoperatieve immuunsuppressie fungeert. Een postoperatieve suppressie werd in beide groepen opgemerkt, maar in de conventionele groep bleef de HLA-DR-expressie onderdrukt tot tenminste vier postoperatieve dagen. De postoperatieve leukocytenreactie was gelijk in de twee groepen.

In *hoofdstuk 3* bestudeerden wij het effect van een chirurgisch trauma (laparoscopische versus conventionele Nissen fundoplication) op het angiogene evenwicht in de circulatie en op het immuunsysteem. De VEGF concentratie in plasma was twee uur postoperatief beduidend lager in de laparoscopische groep en keerde na 24 uur terug naar de preoperatieve waarde. In de conventionele groep was de VEGF plasmaconcentratie na vier postoperatieve dagen significant toegenomen. De concentratie witte bloedcellen en C-reactief proteïne stegen postoperatief snel, waarbij C-reactief proteïne in de conventionele groep sneller toenam dan in de laparoscopische groep. De endostatine concentratie in plasma bleef hetzelfde over de gehele periode in beide groepen. Opnieuw heeft de grootte van het chirurgische trauma gevolgen voor de angiogene en immunologische reacties.

In *hoofdstuk 4* onderzochten wij VEGF- en endostatine concentraties in wondvocht en plasma van patiënten met borstkanker en vrouw-naar-man mastectomie. Wij vergeleken het angiogene profiel tussen waarden in wondvocht en in plasma. In beide groepen waren de VEGF-concentraties in wondvocht veel hoger dan in plasma. Een soortgelijke trend werd ook gevonden voor pro-inflammatoire cytokinen. De endostatine concentratie in wondvocht was lager dan in plasma en nam progressief af. De locale VEGF-toename en endostatine afname meteen na een chirurgisch ingreep, lijkt een fysiologisch reactie te zijn op een trauma. Deze

postoperatieve 'angiogenic switch' werd niet door het soort proces (maligne of benigne) beïnvloed.

In *hoofdstuk 5* onderzochten wij het lokale en systemisch angiogene profiel bij darmkanker patiënten die een curatieve laparoscopische of conventionele ingreep ondergingen. Net als in *hoofdstuk 4* was de VEGF-concentratie in wondvocht beduidend hoger dan de postoperatieve VEGF-concentratie in plasma, terwijl de endostatine waarden in wondvocht lager waren dan waarden in plasma, en postoperatief progressief afnamen in beide groepen. Op de vierde postoperatieve dag was de VEGF-concentratie in wondvocht hoger in de laparoscopische groep dan in de conventionele groep. Dit zou verklaard kunnen worden door de gevolgen van lokale acidose, tengevolge van CO_2-insufflatie, op endotheel cellen.

In de laatste twee hoofdstukken evalueerden we de angiogene balans in het bloed van patiënten die perioperatief behandeld werden met een immunomodulerend middel. Het is bekend dat GM-CSF zowel het immuunsysteem als de angiogenese stimuleert.

In *hoofdstuk 6* onderzochten wij de gecombineerde effecten van chirurgie en rhGM-CSF, op angiogene parameters bij patiënten met darmkanker. In deze fase II ge-randomiseerde, placebo-gecontroleerde studie werden patiënten perioperatief behandeld met rhGM-CSF (2,8 mg/kg lichaamsgewicht) of met fysiologisch zout. rhGM-CSF versterkte de productie van IL-6 en VEGF, maar had geen invloed op endostatine en angiostatine. De chirurgische ingreep induceerde een kortdurende endostatine afname. In beide groepen verschenen na de ingreep twee soorten angiostatine (kringle 1-3 en kringle 1-4). K1-4 angiostatine werd manifest in bijna alle patiënten en K1-3 angiostatine nam in 6 van de 16 patiënten toe. Een mogelijke verklaring hiervoor is dat proteolytische enzymen, die in het vroege wondgenezingsstadium actief worden, ook betrokken zijn in de conversie van plasminogeen naar angiostatine. Samenvattend wordt de angiogenese in een vroeg postoperatief stadium geïnitieerd door toename van VEGF en afname van een angiogenese-remmer (endostatine), gevolgd door de

verschijning van twee angiostatine banden. Dit patroon bevestigt dat het wondgenezingsproces bij kankerpatiënten fysiologisch verloopt. Postoperatieve infectueuze complicaties kunnen mogelijk worden verminderd door het bactericidal permeability increasing protein (BPI), dat lipopolysaccharide (LPS) neutraliseert. Bovendien is aangetoond dat BPI een anti-angiogene werking heeft.

In *hoofdstuk 7* werden patiënten met levermetastasen bij darmkanker perioperatief behandeld met rBPI21 of een placebo. De pro-angiogene factoren IL-6, VEGF en de anti-angiogene factor endostatine werden gemeten. In beide groepen werden in de eerste 24 uur de hoogste IL-6 concentraties gemeten met een piek twee uur na de ingreep. In beide groepen werd tevens in de eerste postoperatieve uren een sterke VEGF-daling waargenomen en in de dagen erna nam de VEGF-concentratie toe. De VEGF-afname zou mogelijk verklaard kunnen worden door een acute expressie van VEGF-receptoren, waardoor circulerend VEGF wordt weggevangen. De toename van VEGF daarna wordt geproduceerd door alle cellen die betrokken zijn in het wondgenezingsproces. Recombinant BPI_{21} had marginale invloed op IL-6 en VEGF. Ook in beide groepen werd een acute endostatine afname waargenomen.

Sammenvattend start een chirurgisch trauma een cascade van complexe humorale en cellulaire veranderingen die zijn gastheer beschermen en de wondgenezing bevorderen. Bij een wond zal een tijdelijke disbalans ontstaan tussen angiogene stimulatoren en remmers. In dit proefschrift demonstreerden wij dat wondgenezing gepaard gaat met een sterke lokale pro-angiogene (VEGF) toename en lokale afname van een anti-angiogene factor (endostatine), wat lijkt op een lokale "angiogenic switch". Dit fenomeen treedt op na een chirurgische ingreep bij zowel patiënten met als zonder kanker en wordt niet beïnvloed door het gebruik van perioperatieve immunomodulatoren. Door het tegelijk bestuderen van wondvocht en plasma toonden we aan dat zowel de immunologische als de angiogene reacties meer uitgesproken zijn in wondvocht dan in de circulatie. Bestudering van het

wondgenezingsproces via het wondvocht levert kennelijk betere informatie op dan bestudering van factoren in het bloed. Tevens demonstreerden wij dat zowel de immunologische als de angiogene reacties onmiddellijk na een verwonding beginnen. Het is interessant de biologische betekenis van lokale en circulerende angiogene factoren na verschillende chirurgische interventies te onderzoeken. Bijvoorbeeld na een laparoscopische of conventionele ingreep, in verschillende organen of gebieden van het lichaam, de invloeden van anesthesie en bij verschillende typen en stadia van kanker. De consequenties voor de tumorbiologie zijn hierbij bijzonder interessant. Het gebruik van anti-angiogene therapie tijdens een chirurgische ingreep valt te overwegen. De combinatie van anti-angiogene therapie en chirurgie zou negatieve gevolgen kunnen hebben voor de wondgenezing, maar tegelijkertijd heilzaam kunnen zijn voor patiënten met kanker. De verschillende angiogenese remmers zullen mogelijk in hun gevolgen voor wondgenezing en anti-tumor activiteit variëren. Bij het gebruik van deze remmers tijdens chirurgie is optimale timing van groot belang. Vanzelfsprekend zijn er meer studies nodig om het angiogenese proces tijdens wondgenezing verder te doorgronden en de mogelijke biologische effecten van angiogene factoren, die door een chirurgisch ingreep gegenereerd worden, op tumorbiologie in kaart te brengen.

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